ELECTROCHEMICAL AND MASS SPECTROMETRIC INVESTIGATIONS OF BIOLOGICAL MOLECULES

By

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1994

To the memory of my mother Sylvia Thompson

"We have only a memory dear wife and mother to cherish our whole life through"

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Sincere thanks go to my parents for their support and guidance. Thanks to my father for his encouragement and understanding throughout the years. Special thanks go to my mother for all her love, she will be always be in my heart. Thanks go to my brothers Errol, Courtney, Peter, Karl and my sister Allison for being there and never saying no. I would like to thank Suzette Riley for her friendship, encouragement and support.

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ELECTROCHEMICAL AND MASS SPECTROMETRIC INVESTIGATIONS OF BIOLOGICAL MOLECULES

By

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Major Department: Chemistry

The chemical characterization of biological molecules is very important to gain insights into the functions of biological systems. Over the past two decades mass spectrometry has become one of the most widely used tools in analytical chemistry. Electrochemistry has a long history in the characterization of redox reactions of many biological molecules. The purpose of this work was to use mass spectrometry combined with gas chromatography (GC/MS) in the detection of 21 protein amino acids, and electrochemistry and mass spectrometry to investigate the reductive activation of the anticancer drug, mitomycin C.

In work study the one-step derivatization of amino acids was achieved using trifluoroacetic anhydride (TFAA) and its homologs, and trifluoroethanol (TFE) and its homologs. The electrophilic derivatives formed are highly sensitive to detection by electron

capture negative ionization MS (ECNI-MS). The one step formation of derivatives has an advantage over techniques previously used. Also, the potential for the formation of cyclic derivatives is another benefit, as these derivatives could yield abundant molecular anions under ECNI conditions providing sensitivity and selectivity for detection by selected ion monitoring.

Electrochemistry on-line with mass spectrometry (EC/MS) has been shown to model the *in vivo* enzymatic oxidation of purines. In this work EC/MS was used to reductively activate mitomycin C by electrochemistry and to detect by on-line mass spectrometry the adducts formed between the electrophillic intermediate of mitomycin C and the DNA base guanosine.

An electrochemical cell based on a thin layer cell design was also developed, this cell allows for the resurfacing and replacement of the electrode which is a major advantage over the electrochemical cell used previously with on-line EC/MS. Off-line electrochemical investigations of mitomycin C in different solvents and at different graphite electrodes were also performed. These experiments were important to ascertain the ideal parameters for the on-line reduction and detection of mitomycin C

CHAPTER 1
INTRODUCTION

The electrochemical and mass spectrometric (MS) investigations of biological molecules are described in this dissertation. The chemical characterization of biological molecules is very important to gain insights into the functions of biological systems. Mass spectrometry combined with gas chromatography (GC/MS), used in the detection of protein amino acids, following one-step derivatization of the amino acid with fluorinated reagents will be described. The electrophilic derivatives formed are highly sensitive to detection by electron capture negative ionization MS (ECNI-MS). The use of electrochemistry combined on-line with a mass spectrometer via a thermospray interface (EC/TSP/MS) to investigate the reductive activation of the anticancer drug mitomycin C (MC) is also described.

The development of an electrochemical cell for on-line EC/MS is also discussed. The electrochemical cell is based on a thin layer design and allows for the resurfacing and replacement of the working electrode. Off-line electrochemical investigations of MC will also be described in this disseration. These experiments were important to determine the ideal parameters for on-line MC reduction and MS detection.

This introductory chapter describes the background of amino acid MS analysis and gives some discussion of previous studies of amino acids by GC/MS. This is followed by an discussion of the electrochemistry of MC. The chapter then concludes with a description of previous studies of biological molecules using EC/MS along with a description of the thermospray and electrospray ionization processes.

Mass Spectrometric Analysis of Amino Acids

Background to Amino Acid Chemistry and Function

Amino acids are substances from which proteins are synthesized.[1] All proteins are composed of 20 'standard' amino acids known as α-amino acids, where a primary amino group and a carboxylic acid group are on the same carbon atom.[2] A side chain gives the amino acids their chemical identity. Many amino acids are not components of proteins. Amino acids are also metabolites of biological energetic reactions, and many are essential nutrients. They function as chemical messengers in communication between cells, e.g. glycine, γ-amino butyric acid (GABA) and dopamine, as intermediates of various metabolic processes, e.g. citrulline and ornithine which are intermediates of urea metabolism. Over 250 amino acids have been isolated from plants and fungi. Many of these are toxic, suggesting they have a protective function in nature. Some, such as azaserine, are medically useful antibiotics.

Amino acid analysis in general has received much attention because of its importance in biological science. Profiling of free amino acids is of great importance and has found broad clinical applications. In the 1950s amino acid analysis was used to diagnose heredity disorders of amino acids metabolism.[3] Amino acid analysis is used in other processes such as low and high protein dietetic production, the determination of a degree of malnourishment, body radiation dose and also the investigation of liver and kidney metabolites. Detection of amino acids are routine in blood serum and plasma,[4] urine and to a lesser extent breast milk, sweat and sperm.[5]

Different separation techniques, based on the charge or hydrophobicity differences in amino acids, are used for both qualitative and quantitative analysis of amino acids. The most common approach in the analysis of amino acid content is the amino acid analyzer.[4] Manual hydrolysis of polypeptides or proteins is followed by high pressure liquid chromatography (HPLC) and on-line post-column derivatization with color or florescent-enhancing derivatizing reagents. Although acid hydrolysis can be slow, chromatographic analysis is rapid and works well for protein amino acids. Unusual nonprotein amino acids are often not easily identified. Sensitivity in HPLC based amino acid analysis is often a problem. Other techniques such as gas chromatography (GC) and mass spectrometry (MS) has been used to analyze amino acids and can achieve greater sensitivity.

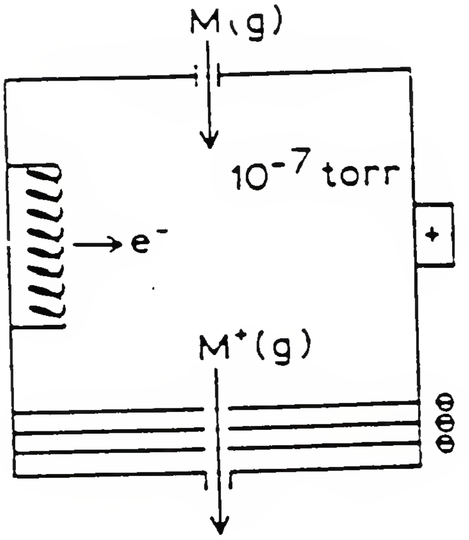
Mass Spectrometry of Amino Acids

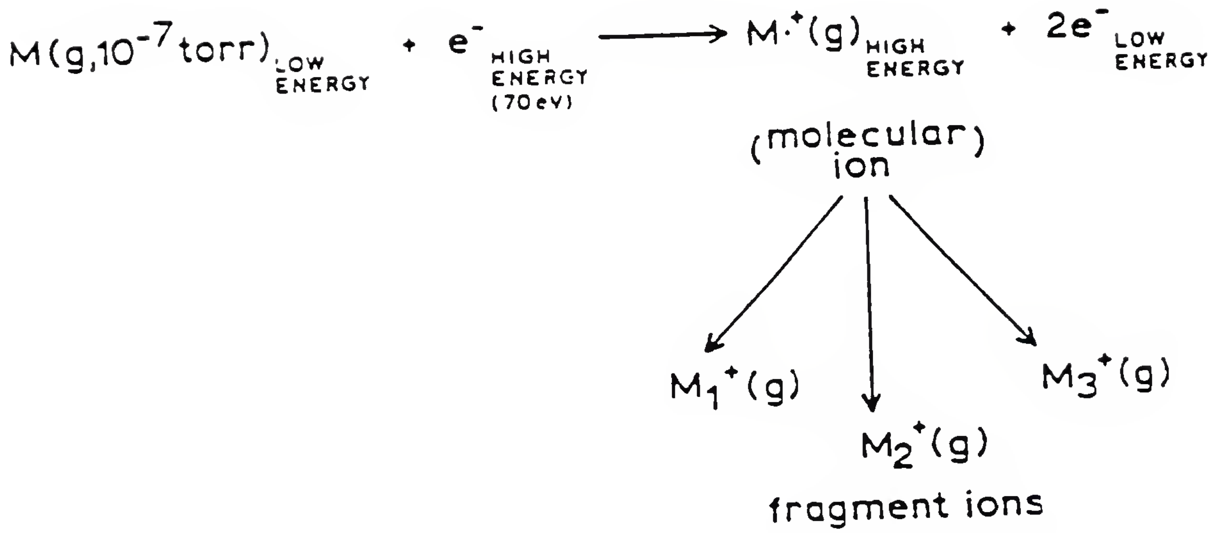
Mass spectrometry is a powerful technique that allows for unambiguous structural verification and identification of many substances including amino acids[5]. Electron impact (EI) mass spectrometry, where electron beam bombardment is used to ionize gas phase molecules, was the first ionization technique used in mass spectrometry of amino acids. Figure 1.1 schematically illustrates electron impact ionization (EI), where ionization of the gas phase sample molecule (M) is induced by collision with a high energy electron beam (ca. 70 eV). Removal of an electron from the sample molecule, M, produces a radical molecular ion ($M^{+\cdot}$). Due to the high energy involved in this process the radical molecular ion undergoes decomposition to give fragment ions (Figure 1.1).

Two modes of sample introduction were used for EI mass spectrometry, direct inlet system and gas chromatography. A direct inlet system was used to analyze free amino acids

Figure 1.1: Illustration of Electron Impact Ionization
A) Schematic Representation of the Ion Source.
B) Gas Phase Reaction Scheme.

ELECTRON IMPACT (EI)



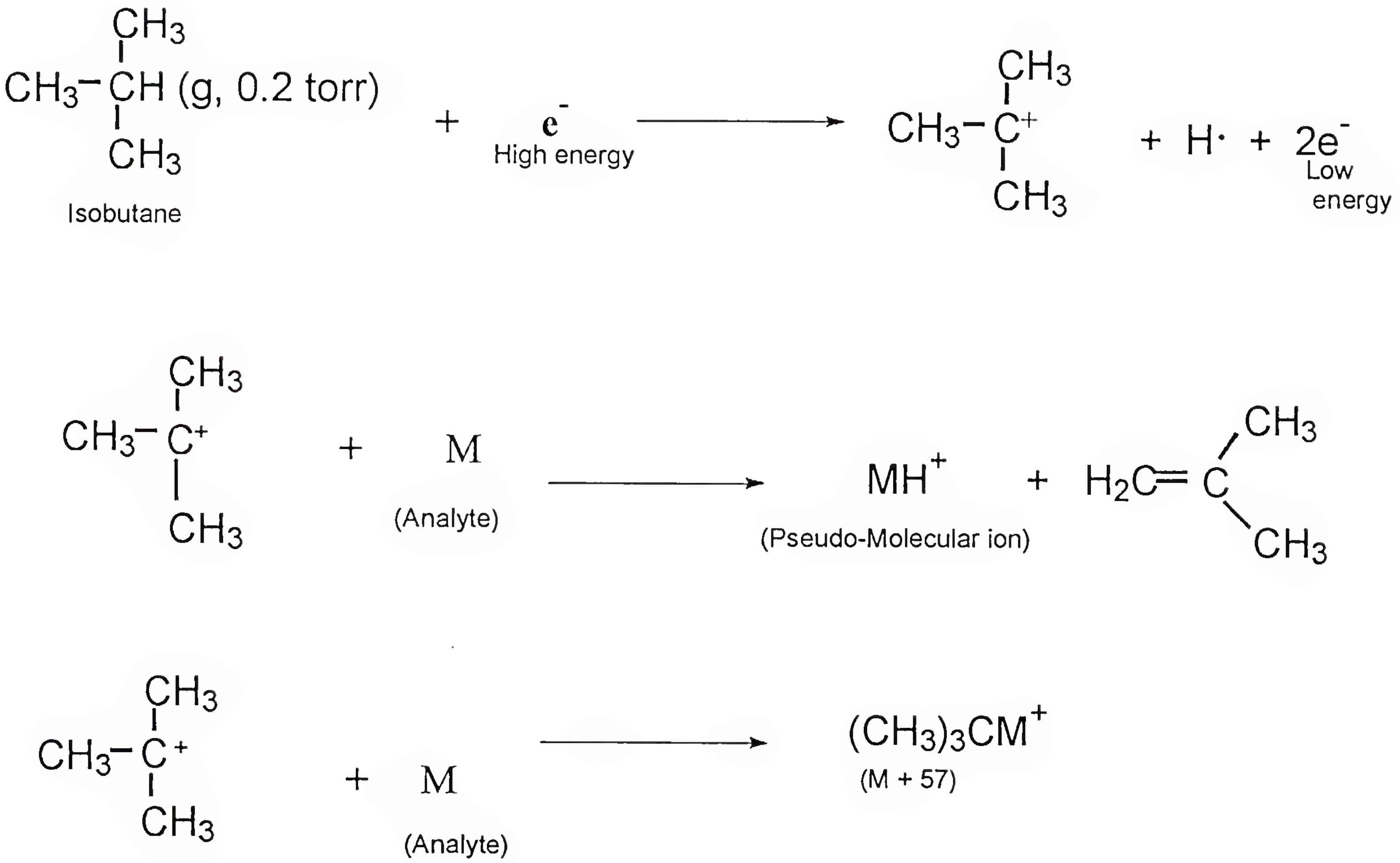

M(g, 10^{-7} torr) LOW ENERGY + e^- HIGH ENERGY (70 eV) → $M^{\cdot +}(g)$ HIGH ENERGY + $2e^-$ LOW ENERGY
(molecular ion)
$M_1^+(g)$
$M_2^+(g)$
$M_3^+(g)$
fragment ions

and amino acid derivatives. The development of GC/MS allowed for the separation and detection of amino acids in complex matrices which previously required tedious, time consuming and difficult sample clean-up.

The high energy transfer encountered during EI conditions produces an abundance of fragmentation and ion rearrangement. This fragmentation and rearrangement makes molecular weight determination difficult, as molecular ion peaks are almost always absent or at very low intensity in EI-MS. Molecular ions can be obtained using different ionization methods. Chemical ionization (CI), field ionization (FI) and field desorption (FD) allow for the molecular weight determination due to the abundance of molecular ions.[3] This is important in the identification of the unknown amino acids and the analysis of amino acid mixtures. In contrast to EI these ionization techniques give little or no structural information due to the lack of fragmentation.

Chemical ionization (CI) was developed to ionize molecules with a much reduced energy transfer, as compared to EI, thus giving stable molecular ions.[6] In this method a reagent gas is ionized at an ion source pressure of about 0.1 kPa and the reactive ionized plasma is used to ionize sample molecules by ion molecule reactions. Figure 1.2 illustrates the ionization sequence involved in CI with isobutane as the reagent gas. Isobutane is ionized giving the principal EI product. This product reacts with sample molecules (M) by proton transfer producing protonated molecular ions ($M+H^+$) which are called quasi-molecular ions. Additional products may also arise as the isobutane ion sample molecule adducts.

Figure 1.2: Chemical Ionization Reactions with Isobutane as the Reagent Gas.


CH3
CH3–CH (g, 0.2 torr)
CH3
Isobutane
e-
High energy
CH3–C+
CH3
CH3
+ H· + 2e-
Low energy
CH3–C+
M
(Analyte)
MH+
(Pseudo-Molecular ion)
H2C═C
CH3
CH3
(CH3)3CM+
(M + 57)

CI-MS as EI can be done directly in the mass spectrometer ion source or in combination with gas chromatography (GC). Free protein amino acids and their methyl esterand N-acetyl derivatives have been analyzed using both direct introduction and GC. Field desorption (FD) MS of free protein amino acids was first performed by Winkler and Bleakey.[7] FD-MS analysis of amino acids has its limitations because it cannot be combined with a separation technique such as GC.

Amino acids are not stable at elevated temperatures and thus require conversion to volatile, thermally stable derivatives before they can be analyzed by GC. Amino acid derivatization generally involves esterification of carboxyl groups with alcohols and acylation of amino groups with halogenated anhydrides.[5] The most common amino acid derivatives used in GC analysis are tert-butyl dimethylsilyl (TBDMS) and the N-trifluoroacetyl butyl ester (BTFA). All protein amino acids can be analyzed by GC after derivatization with these compounds.[8]

Gas chromatography combined with mass spectrometry offers the possibility of the unequivocal identification of amino acids even at nanomolar quantities.[5] Unequivocal and reliable identification of known and unknown amino acids even in complex mixtures can be accomplished with the use of mass spectrometry. Selectivity and sensitivity of mass spectrometry allows for accurate mass measurement with the aid of selected ion monitoring (SIM).

In the SIM mode selected individual ion masses of the compound to be analyzed are monitored continuously, or if several ions are measured, each is detected in sequence for a fixed period of time. By continuously monitoring one or a few ions, preferably the molecular ion, instead of measuring several hundred ions in a complete spectrum, the limit of detection is increased by a factor up to 100 or possibily even more.

SIM reduces chemical noise and can be used for trace analysis. Chemical ionization (CI) mass spectrometry is ideally suited for SIM analysis, because of the soft ionization with little or no fragmentation which allows the molecular ions to be easily detected [4].

Derivatives of Amino Acids for GC/MS Analysis

Amino acids are non-volatile and must first be derivatized to volatile compounds for GC/MS analysis. The carboxylic acid group is converted to an ester and the amine group is acylated to an anhydride. N-perfluoroacyl alkyl esters (TAB) and trimethylsilyl (TMS) derivatives are the most popular for the quantitative analysis of amino acids.[7]

TMS derivatives have been formed from various types of amino acids.[9] All amino acids except arginine have been quantitatively derivatized for GC analysis, sometimes with drastic reaction conditions.[10] However, the derivatives are very easily hydrolyzed and require well-preheated GC columns to avoid loss of the TMS groups. Therefore, they are considered less suitable for GC/MS analysis than the TAB amino acid derivatives.

Gehre and coworkers used trifluoroacetyl (TFA) n-butyl esters (TAB amino acids) for quantitative analysis by GC.[11] This was extended to GC/MS analysis by Duffield and coworkers.[12] The two-step synthesis involves the esterification of the amino acid's carboxyl groups with n-butanol. The amino and hydroxyl groups of the resulting ester are acylated with trifluoroacetic anhydride (TFAA), or a higher homolog such as pentafluoropropionic anhydride (PFPA), producing N-TFA n-butyl or N-PFP n-butyl esters.

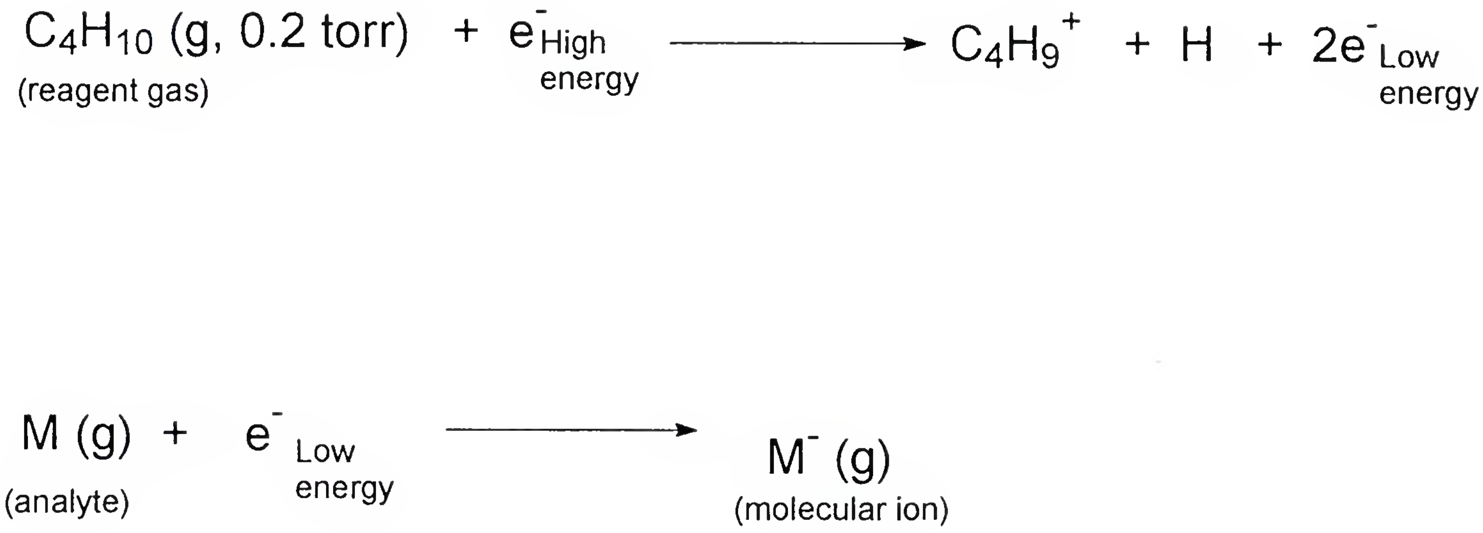
TAB amino acid derivatives have been used in GC/MS analysis of 22 free proteins amino acids.[8] TAB analysis was also extended to the detection of bacterial amino acids in environmental samples, body fluids and tissues.[4] Another clinical chemistry application of TAB amino acids and GC/MS analysis was the detection of the amino acids composition of streptococcal peptidoglycan-polysaccharide complexes.[4] The amino acids were analyzed as the butyl hepta fluorobutyl derivatives with selected ion monitoring (SIM) which enabled the amino acids to be analyzed without interference from background noise. Amino acids in brain and plasma samples were analyzed by GC/MS with pentafluoropropionic anhydride and hexafluoroisopropanol derivatives.[5]

Trifluoroacetic anhydride (TFAA), in addition to its use as an acylating reagent for alcohols and amines, also promotes rapid esterification of organic and inorganic oxyacids, such as amino acids, by alcohol via a three-step pathway.[13,14] This catalytic property has been exploited for the development of a one-step acylation/esterification procedure for polyfunctional molecules. The anhydride serves both to acylate the amino and the hydroxyl groups and to catalyze the esterification of the oxyacid groups by an alcohol. This single-step acylation/esterification procedure has been applied to amino acids. Para-aminobenzoyl glutamic acid (pABG), an end product of the metabolism of folic acid in humans, was analyzed by GC/MS using the one-step acylation/eterification procedure.[15] Also pentafluoropropanoic anhydride (PFPA)/pentafluoropropanol (PFP) mixtures were used to derivatize ten gamma-glutamyl amino acids for GC analysis.[16]

Electron Capture Negative Ion Mass Spectrometry (ECNI-MS)

The development of CI applications to biological compounds has renewed interest in negative ion chemical ionization (NICI) MS[17]. Negative ions can be formed in the ion

Figure 1.3: Electron Capture Reactions (ECNI)



$$\underset{\text{(reagent gas)}}{C_4H_{10}\ (g,\ 0.2\ \text{torr})} + e^-_{\substack{\text{High}\\\text{energy}}} \longrightarrow C_4H_9^+ + H + 2e^-_{\substack{\text{Low}\\\text{energy}}}$$

$$\underset{\text{(analyte)}}{M\ (g)} + e^-_{\substack{\text{Low}\\\text{energy}}} \longrightarrow \underset{\text{(molecular ion)}}{M^-\ (g)}$$

source of the mass spectrometer by resonance electron capture. Figure 1.3 illustrates how this can be achieved in a CI ion source where the reagent gas removes the excess energy from the high energy electron beam, producing low energy electrons (near thermal energy) that are captured by the sample molecule (M). The introduction of electrophilic groups such as fluorine to amino acids enhances their electron affinity and produces derivatives that exploit the high sensitivity and selectivity of the electron capture negative ion (ECNI) mass spectrometry. For successful utilization of ECNI mass spectrometry the target molecule must have a large cross-section for electron capture.[18,19] If the analyte has an electronegative center e.g., Cl, F, Br_2, or NO_2 the sensitivity of ECNI-MS is enhanced at least 100 fold versus electron impact ionization. If the analyte has no electronegative center, there is no enhancement in sensitivity versus electron impact ionization.

ENCI-MS has been successfully used for the analysis of trace metals in the atmosphere, the analysis of polychlorobenzodioxins and related compounds in biological samples, and the analysis of drugs and drug metabolites in biological samples.[20] Low and Duffield have applied ECNI-MS to the analysis of amino acids. Carboxy-n-butyl ester N-(O,S)-pentafluoropropionate derivatives of 29 amino acids were analyzed and their mass spectra determined. Positional isomers within the same class gave different NICI mass spectra, while the positive ion (PI) mass spectra could not distinguish these isomers. Increased sensitivity was also observed for ECNI over PI, with derivatized leucine being 25 times more sensitive in ECNI-MS.[21]

Mitomycin C

Background and Properties

Mitomycins, a group of antitumor antibiotics were isolated in 1956 by Hata and coworkers.[22] They were extracted and then purified by chromatography from the fermentation broth filtrate of *streptomyces caespitosus.* Mitomycin C (MC) was isolated in 1958 by Wakaki and co-workers.[23] Mitomycin C is an antineoplastic agent and has been used in the treatment of several types of cancers including superficial cancer of the urinary bladder and combinations of lung, breast and stomach cancers.[24]

The molecular formula of mitomycin C is $C_{15}H_{18}N_4O_5$ and the molecular weight is 334.13. The structure of MC is shown in Figure 1.4. The IUPAC name of mitomycin C is [1a, R]-6-amino-8[[(aminocarbonyl)oxy]methyl]- 1, 1a, 2, 8, 8a, 8b-hexahydro-8a-methoxy-5-methyl azirino [2, 3', 3, 4] pyrrolo [1.2-9] indole-4-7- dione. Mitomycin C is marketed under the trade names Mutamycin, Mitomycin-C Kyowa and Ametycine.[23] It is an odorless blue-violet crystalline powder.

Drug Activity

MC anticancer activity is expressed by its ability to alkylate DNA with cross-linked covalent bonds. To exhibit this intracellular cancer fighting ability, reduction of the quinone moiety of MC by enzymes is essential.[25] The major bioreductive catalyst for MC activation is cytochrome P-450 reductase, a membrane bound flavoenzyme.[26] Chemical reducing agents such as sodium dithionite,[27] and electrochemical reductions on mercury and platinum electrodes[28] have been used for MC reductive activation in vitro.

The theoretical mechanism for the reduction of MC *in vivo* (Figure 1.5) was first

Figure 1.4: Structure of Mitomycin C

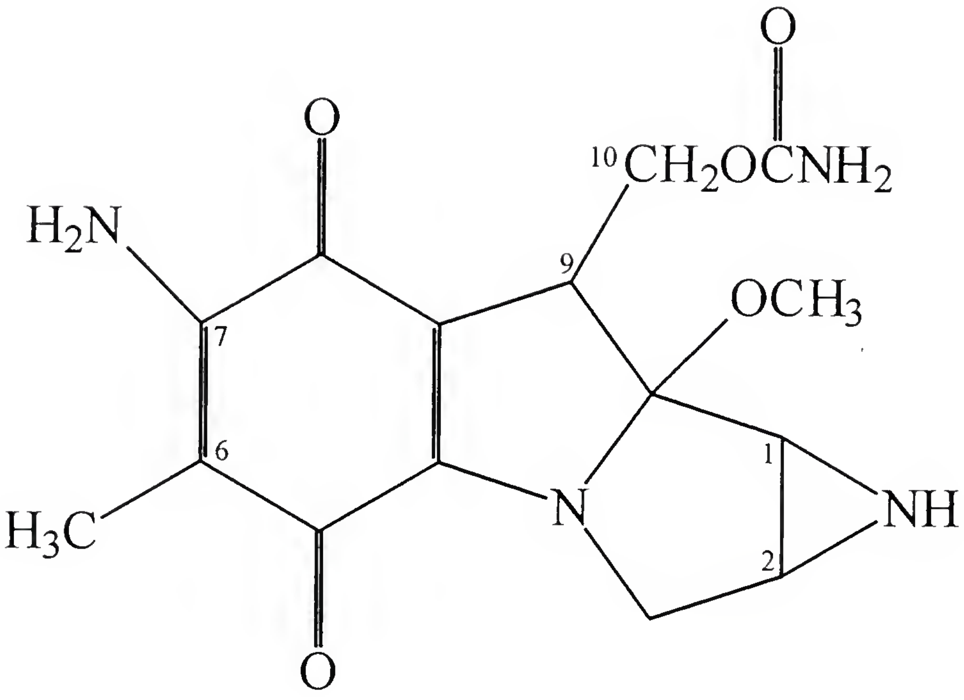
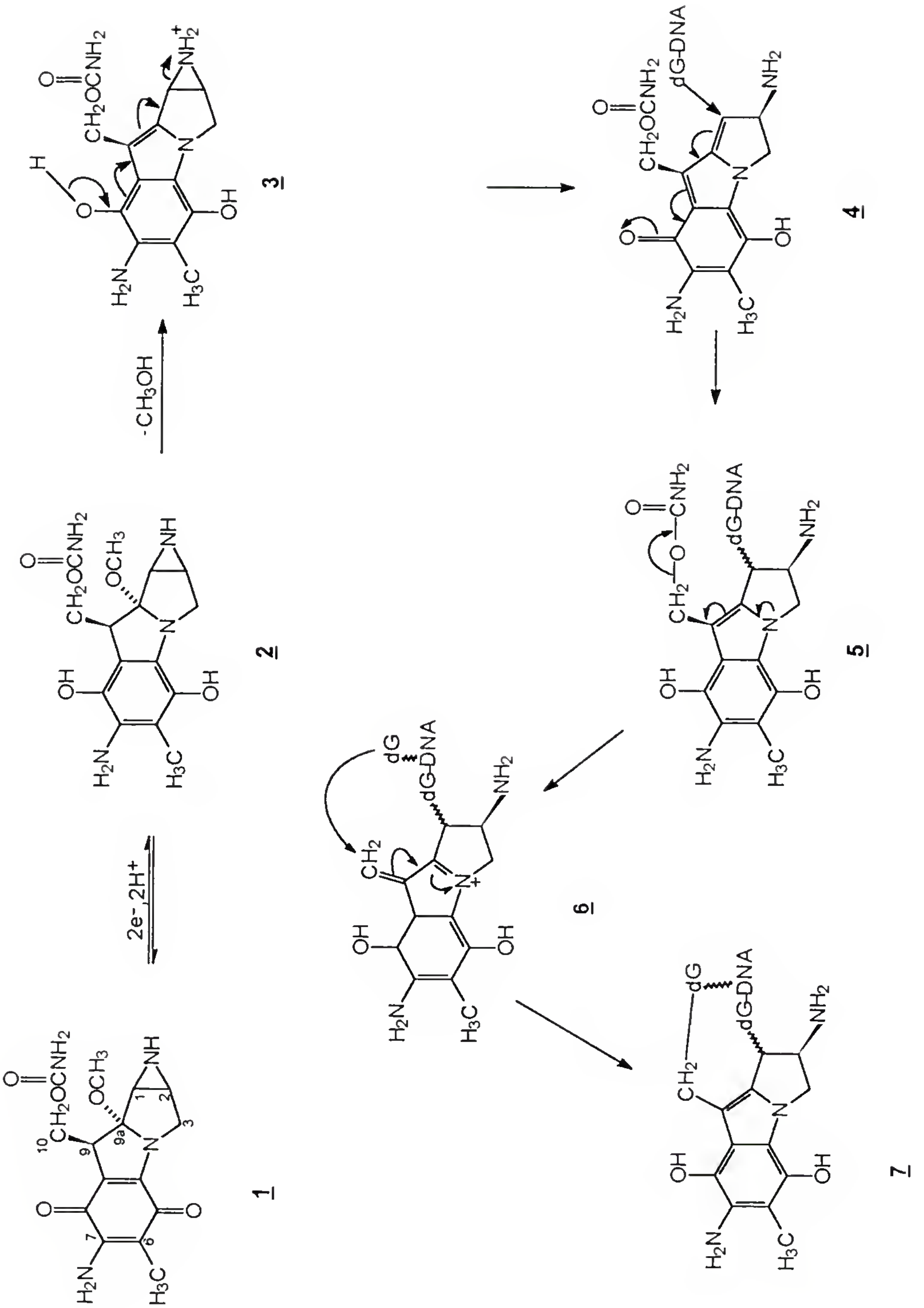

O
O
$^{10}CH_2OCNH_2$
H_2N
9
OCH_3
7
6
1
N
NH
H_3C
2
O

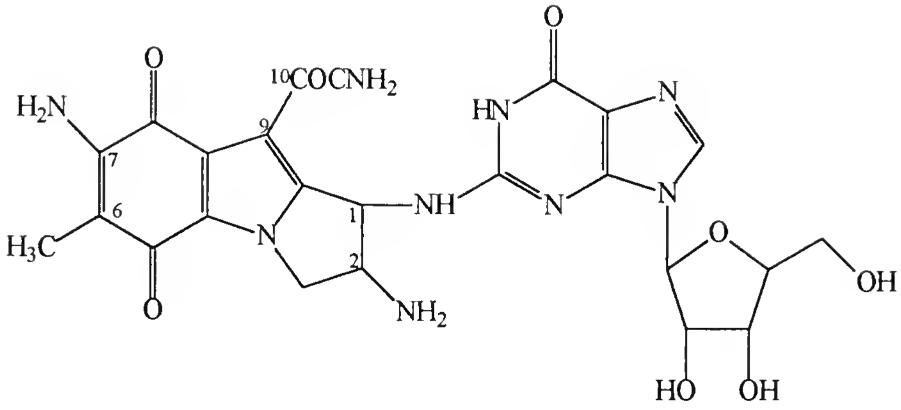
Figure 1.5: Proposed Mechanism of Mitomycin C Drug Function.[29,30]


1
2
3
4
5
6
7
2e⁻, 2H⁺
- CH3OH
CH2OCNH2
OCH3
NH
NH2+
H2N
H3C
OH
N
dG-DNA
dG
CH2
NH2
10
9
9a
1
2
3
7
6

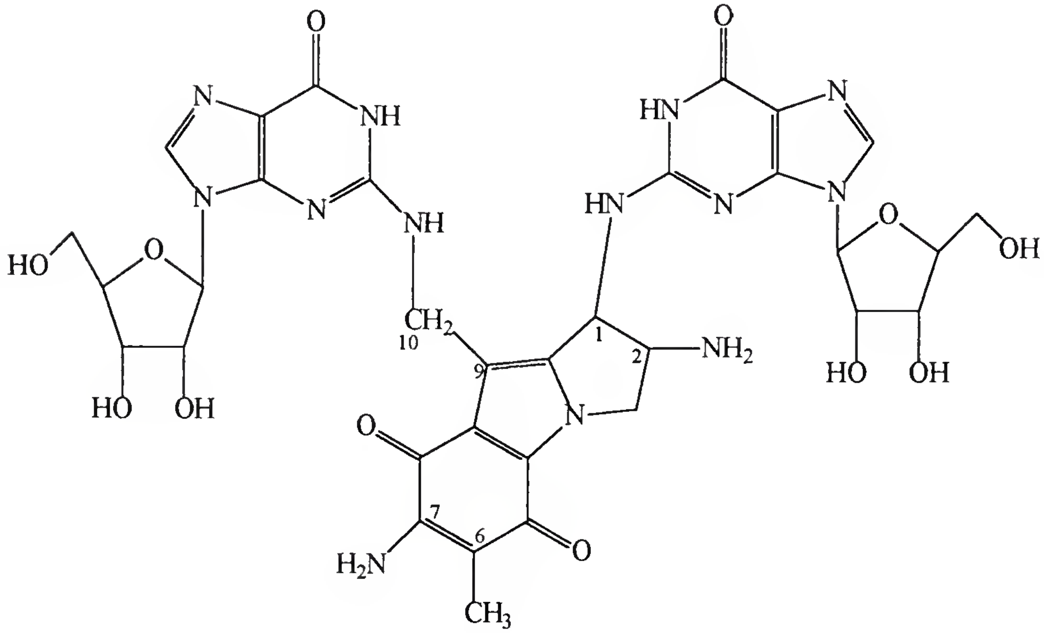
described by Iyer and Szybalski[29] then later by Moore.[30] According to the mechanism proposed by Moore, MC **1** is reduced *in vivo* to the hydroquinone **2**, followed by the elimination of methanol forming **3**, and the opening of the sterically strained three-membered aziridine ring forming **4**. This gives the biologically active form of MC **(4)** which can react with DNA. DNA, acting as a nucleophile, can attack at the C-1 active site forming **5**. The C-10 position is also a possible site for the nucleophilic attack, and a crosslinked DNA adduct **7** can be formed. The MC-DNA monoadduct **5** formation occurs on the exocyclic N-2 position of the deoxyguanosine base of the DNA and the C-1 position of the MC. The crosslinked adduct **7** has an additional deoxyguanosine from the same DNA molecule attached at the MC C-10 position. The interaction of MC with DNA produces more than 90% of the monofunctional adduct.[24]

Both the monofunctional and crosslinked adduct of MC and the DNA-base guanosine have been isolated and their chemical structures reported. The major monofunctional adduct was isolated from a MC-DNA complex and characterized by Tomasz and co-workers[25] as N^2-(2" β,7"-diaminomitosen-1"α-yl)-2'-deoxyguanosine (Figure 1.6a). The crosslinked (bifunctional) adduct (Figure 1.6b) of MC with the DNA was also isolated and characterized by Tomasz and coworkers[25] following the *in vitro* reductive activation of MC. MC was activated by the chemical reducing agents hydrogen/platinum oxide (H_2/PtO_2) and sodium dithionite ($Na_2S_2O_4$) and mixed with DNA and poly(dG-dC) complexes. The reductive activation of MC with hydrogen/platinum lead to a mixture of mono and bifunctional adducts. However, when sodium dithionite was used to reductively activate MC, the alkylation was exclusively bifunctional.

Figure 1.6: Structure of Mitomycin C-DNA Adducts a) Monoadduct b) Bifunctional Adduct.


O
10COCNH2
O
H2N
9
HN
N
7
1
NH
N
N
6
H3C
N
2
O
OH
O
NH2
HO
OH

a


O
O
N
NH
HN
N
N
N
HN
N
N
NH
O
O
HO
OH
CH2
10
1
2
NH2
9
HO
OH
HO
OH
N
O
7
6
O
H2N
CH3

b

The bifunctional adduct was characterized by Tomasz and coworkers with spectroscopic methods that included UV analysis, proton magnetic resonance, FTIR, and circular dichroism.[25] The bifuctional adduct was also isolated from the liver of rats that had been injected with MC. HPLC and UV analysis was used for the identification of the isolated adducts. The identification of these adducts is a direct confirmation of the bifunctional alkylation of DNA by MC and supports the Iyer-Szybalski and the Moore mechanisms.

Mass spectrometric methods have also been used to characterize the MC-DNA adducts. Musser, Pan and Callery used HPLC/thermospray MS to analyze DNA adducts formed from MC and calf thymus DNA.[31] MC was reductively activated by hydrogen gas with PtO_2 catalyst and mixed with calf thymus DNA in tris buffer of pH 7.4. DNA was precipitated and digested to nucleotides and this mixture was analyzed by thermospray/HPLC-MS. The adducts formed from the reaction of MC with DNA were separated from unmodified nucleosides by HPLC on a C18 column. The mobile phase used for analysis was 0.1 M ammonium acetate/5% methanol with a 30 minute linear gradient to 0.1 M ammonium acetate/50% methanol with a flow rate of 1.2 ml/min. The thermospray interface temperatures were optimized at tip temperature of 210-220°C with a block temperature of 295°C. The principal ions in TSP-MS of the MC-deoxyguanosine adduct, MW= 569, were the $(M+H)^+$ molecular ion at 570 daltons and the ion at 244 daltons which was proposed to be a protonated MC fragment.

Tomasz and co-workers[25] used fast atom bombardment mass spectrometry (FAB-MS) to analyze the bifunctional adduct of MC. Analysis was performed in a glycerol matrix with xenon as the ionization gas and a sample size of ca. 0.1 μg. The molecular ion due to the bifunctional adduct was seen at 986 daltons. Fragments at 635, 636, 637, 586, 435, 376, 284 and 242 were identified and the fragmentation pathway was established by MS-MS studies of the monoadduct.

Electrochemical Analysis

The electrochemical reduction of MC at a hanging mercury drop electrode in buffered aqueous solutions has been previously investigated.[32] In the cyclic voltammetric data two successive electrochemical reduction waves were observed at -368 and -468 mV versus SCE respectively. They were attributed to the two-electron, two-proton quinone-hydroquinone reduction of MC and it's ring-opened moiety 1-hydroxy-2,7-diaminomitosene (Figure 1.7). It was proposed that the aziridine ring-opening occurred from the hydroquinone in aqueous media, and the quinone radical intermediate was not detected as is generally the case in aqueous media.

The electrochemical reduction of quinones in polar aprotic media such as DMF and Me_2SO is known to involve the initial formation of an anion radical prior to the formation of the semiquinone and the hydroquinone species.[33] The lipid bilayers of cellular membranes are heterogenous, consisting of both hydrophilic and hydrophobic regions. The reduction of MC may conceivably take place in the lipid matrix where the radical anion may have a considerable lifetime. The aprotic environment favors mechanistic pathways different from those in protic environments. So, the study of MC reduction in DMF and Me_2SO is relevant to both the metabolic activation and the mechanism of action of MC.

The proposed mechanism for the reduction of MC in DMF and Me_2SO is shown in Figure 1.8. The MC radical anion **9** has been reported as stable in the aprotic, anaerobic DMF

Figure 1.7. Structure of Hydroxy Substituted MC Moiety 1-hydroxy-2,7-diaminomitosene

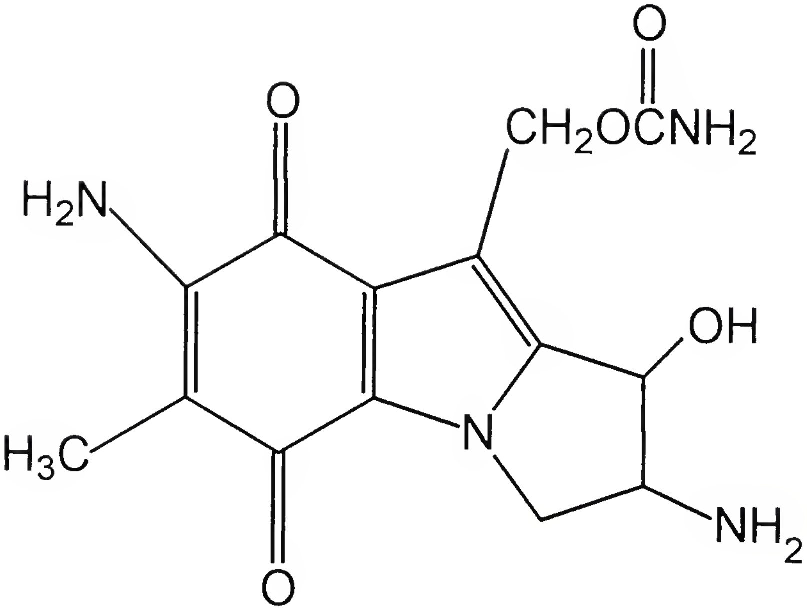
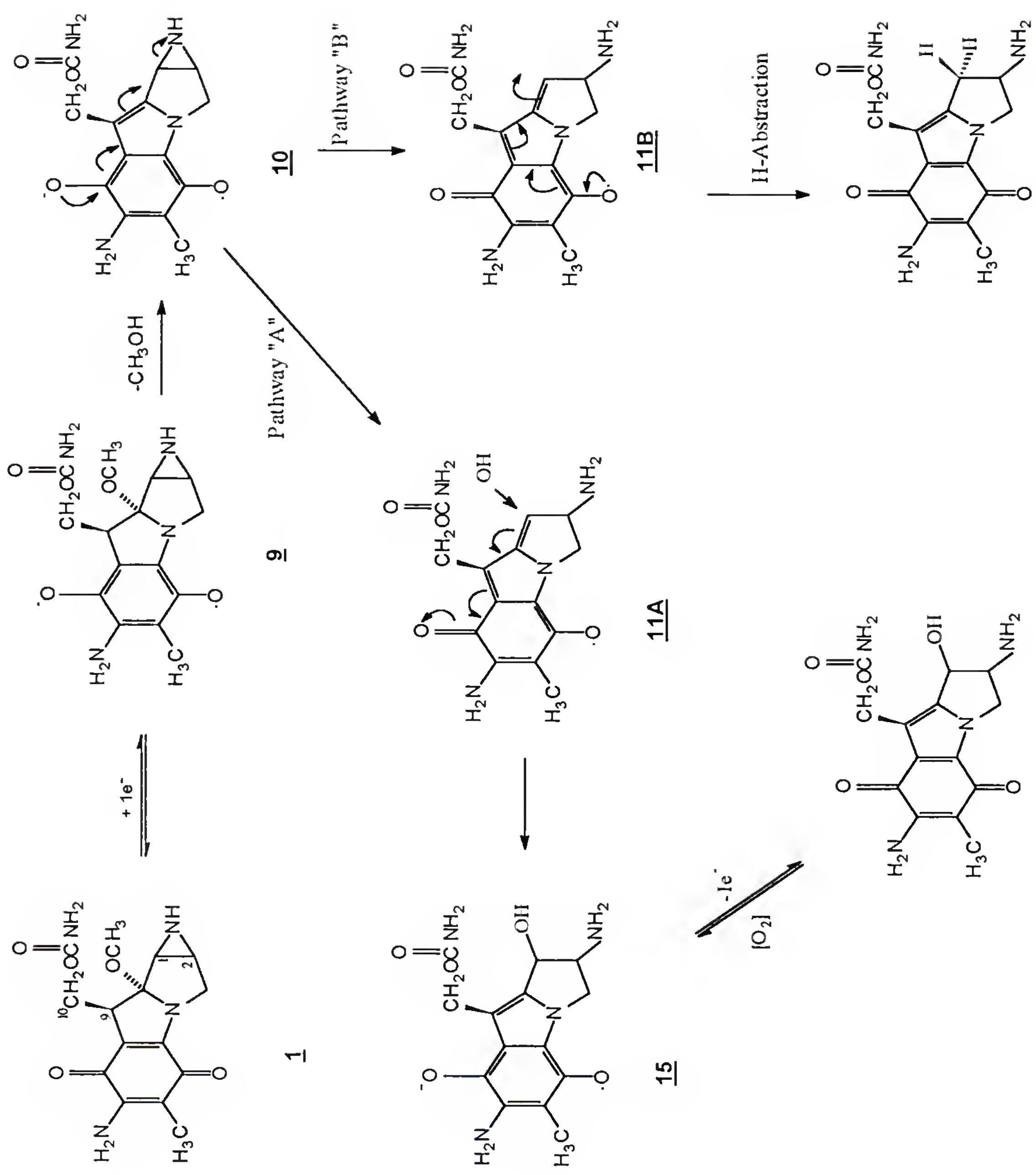

O
H2N
H3C
O
CH2OCNH2
O
OH
N
NH2

Figure 1.8: Proposed Mechanism for the Reduction of Mitomycin C in DMF.[33]

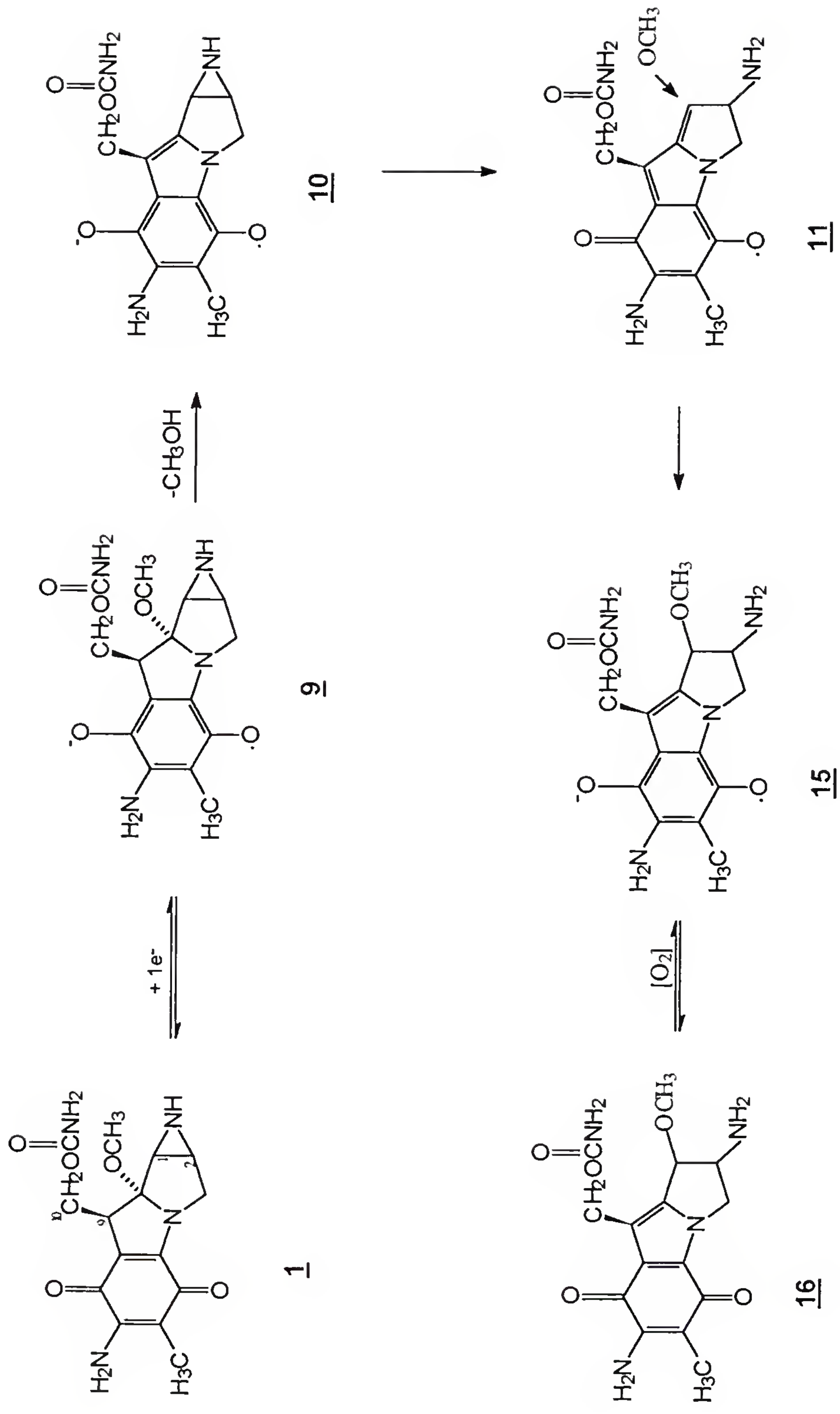

1
+ 1e⁻
9
-CH₃OH
10
Pathway "A"
Pathway "B"
11A
11B
H-Abstraction
12
15
-1e⁻
[O₂]
16

and Me_2SO solutions. However, upon addition of H_2O after the formation of **9**, a complex mixture of products was formed. The addition of water creates a polar environment which favors the loss of methanol forming **10** and the subsequent opening of the three-membered aziridine ring forming **11A**. The attack of the nucleophile OH^- at C-1 leads to **15** and subsequent reoxidation of **15** leads to **16** (pathway "A"). An alternative pathway (pathway "B") was also proposed. The intermediate **11B** generates a carbon centered free radical at C-1 instead of undergoing nucleophilic attack at C-1 position. Hydrogen abstraction from H_2O then takes place and **12** is formed. Pathways A and B are competitive reactions with pathway "A" favored at basic pH due to the increasing availability of OH^- to attack C-1. Products **16** and **12** were isolated and identified and radical formation proposed from the formation of these products.

It was suggested that the one-electron reduction was sufficient to activate MC to reactive intermediates and was the primary mode of the bioreductive activation. This is because the radical anion generated products following the water hydrolysis reactions closely resembling the profile of products from the reduction with cytochrome P-450 reductase.

The electrochemical reduction of MC by cyclic voltammetry at mercury and platinum electrodes was investigated by Kohn and co-workers[29] to determine probable reduction pathways in the protic non-aqueous solvent, methanol, and in buffered aqueous solutions. Figure 1.9 shows the proposed mechanism for the reduction of MC in methanol. It was determined that the activation of MC, that is the loss of methanol forming **10** and the aziridine ring opening forming **11** proceeds from the quinone radical stage **9** which is in agreement with the scheme proposed for MC reduction in DMF (Figure 1.8). There is then

Figure 1.9: Proposed Mechanism for the Reduction of Mitomycin C in Methanol.[28]


1
+ 1e⁻
9
-CH3OH
10
11
15
[O2]
16
H2N
H3C
CH2OCNH2
OCH3
NH
NH2

the addition of the nucleophile $^{-}OCH_3$ to the C-1 position of MC forming **15**. Oxidation of **15** leads to the formation of the quinone **16**.

Electrochemistry On-Line with Mass Spectrometry

Insights into Biological Redox Reactions

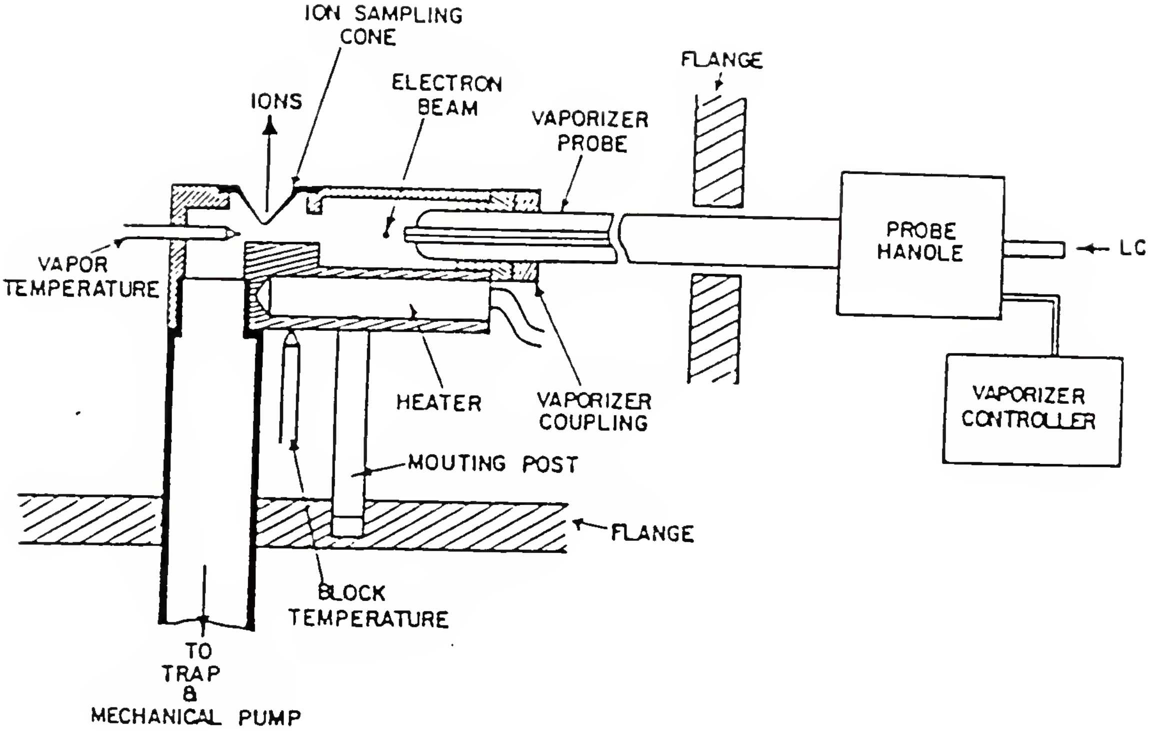
In the past two decades research into biological systems and how they work has increased dramatically. One type of biological reactions that is of interest to electrochemists is redox biological reactions. Reactions at the electrode-solution interface in electrochemical experiments can be used to model enzymatic redox reactions that take place in biological systems. The electrochemical reactions can as a result provide useful information about biological reaction pathways. Enzymes such as peroxidase,[34] cytochrome P-450[35] and xanthine oxidase[36] give products that are chemically similar to those obtained in electrochemical reactions. The pathways of the enzymatic and the electrochemical reactions are also similar. Insights into the enzymatic reactions are most successfully obtained when electrochemical analysis is used in combination with other analytical methods such as HPLC, molecular spectroscopy and mass spectrometry. Kissenger and coworkers used HPLC on-line with an electrochemical cell to obtain insights into redox reactions.[37] Gas chromatography/mass spectrometry (GC/MS) has been used for the analysis of electrochemical products of biologically important reactions.[38] These products are mainly polar, non-volatile compounds that require time consuming, often tedious, derivatization prior to GC/MS analysis. Derivatization limits the ability of GC/MS to identify short-lived intermediates. To detect intermediates by GC/MS spectroelectrochemistry had to be used for fast detection of intermediates which were electrochemically generated then frozen, freeze dried and derivatized for GC/MS analysis.

The development of liquid interfaces for mass spectrometry such as thermospray[39] and more recently electrospray[40] has made it possible for the use of MS for on-line detection of non-volatile, thermally labile biological molecules. The ability to link the solution directly to the mass spectrometer source has proven to be very useful in the analysis of thermally labile, non-volatile compounds such as nucleotides and nucleosides.[41] With the use of liquid interfaces derivatization or extensive sample work-up is not required making LC/MS an excellent method for the rapid identification of biologically formed adducts. The thermospray interface has allowed for the direct coupling of electrochemistry with MS. The on-line combination of MS and electrochemistry (EC/MS) offers the capability to directly monitor reactants, short-lived intermediates and products of electrochemical reactions as a function of electrode potential.[42] The combination of electrochemistry with MS provides a direct, sensitive and highly selective method for detecting individual components in complex biological redox reactions.

Thermospray Ionization

Thermospray (TSP) was developed by Vestal and Blakeley[39] from efforts to make a practical liquid chromatography-mass spectrometry (LC/MS) interface that would allow for the analysis of non-volatile, thermally labile samples in solution. Thermospray has since then become the most widely recognized technique for interfacing LC/MS and is routinely applied to a wide range of samples in solution including volatile and non-volatile, polar and non-polar, labile and stable compounds. A diagram of a thermospray interface is shown in Figure 1.10.

Figure 1.10: Diagram of Thermospray Interface.


ION SAMPLING CONE
IONS
ELECTRON BEAM
VAPORIZER PROBE
FLANGE
PROBE HANOLE
LC
VAPORIZER CONTROLLER
VAPOR TEMPERATURE
HEATER
VAPORIZER COUPLING
MOUTING POST
FLANGE
BLOCK TEMPERATURE
TO TRAP & MECHANICAL PUMP

The principle of thermospray involves the vaporization and ionization of the analyte solution by passing it through a heated metal capillary at a flow rate of approximately 1-2 mL/min. The mechanism of the vaporization process relies on the transfer of heat to nearly complete the vaporization of the liquid. The vaporization process occurs in the source of the mass spectrometer that is under vacuum. The effluent from the capillary vaporizer is a supersonic vapor jet consisting of a superheated mist of fine particles and solvent droplets. Introduction of the vapor into the MS source is critical to the optimum thermospray performance because ionization of the sample occurs in the vapor phase. The best thermospray performance occurs when there is a maximum ionization and this occurs when the vaporization is ca. 95% complete within the capillary tube. It is vital that the heat input is properly controlled so that premature vaporization does not occur deteriorating MS sesitivity.

In the thermospray process sample ions can be produced by gas-phase reactions which resemble chemical ionization (CI). Ammonium acetate ($NH_4CH_3CO_2$) is a typical ionic solute used as a reagent gas in thermospray analysis of aqueous solutions. $NH_4CH_3CO_2$ will ionize in the gas phase to NH_4^+ and CH_3COO^-. A neutral analyte molecule M, in the aqueous $NH_4CH_3CO_2$ mobile phase, can form positive ions by the acid-base gas-phase reaction with the ammonium ion:

$$M + NH_4^+ \dashrightarrow [M+H]^+ + NH_3 \qquad (1)$$

$$M + NH_4^+ \dashrightarrow [M+NH_4]^+ \qquad (2)$$

Reaction 1, the proton transfer, will dominate when the proton affinity of the analyte M is greater than that of the ammonium ion. Adduct formation, reaction 2, dominates when

M has a lower proton affinity than the ammonium ion.

Negative ions can be formed from the TSP reactions of the analyte molecule, M, and the acetate ion. Proton transfer (3) and adduct formation (4) reactions can occur:

$$M + CH_3COO^- \text{ ----> } [M\text{-}H]^- + CH_3COOH \qquad (3)$$

$$M + CH_3COO^- \text{ ----> } [M\text{+}CH_3COO]^- \qquad (4)$$

Hambitizer and Heitbaum were the first to successfully use thermospray to interface an electrochemical cell on-line with a mass spectrometer.[43] In these experiments the electroxidation of N,N-dimethylanaline (DMA) at a Pt electrode was detected on-line by MS. The oxidation products, dimers and trimers of DMA, were detected with a time resolution of ca. 10 seconds between the oxidation in the electrochemical cell and the detection by the mass spectrometer.

The delay time between generation and detection and the dead volume of the transfer lines are important parameters in EC/MS. If they are too large, the time resolution of MS detection will be degraded and the probability of detecting short-lived intermediates will be greatly decreased. Volk and co-workers used EC/MS with a thermospray interface to model the enzymatic redox reactions of purines.[44] With the instrumental system used in these experiments the time resolution was improved to ca. 500 ms, therefore, short-lived intermediates could be detected. The oxidation of uric acid, which was one of the reactions studied, is a simple process, but the chemical reactions in aqueous solution that follow the transfer of electrons are complex leading to the formation of many intermediates and products. Twelve intermediates and products of uric acid were detected using EC/MS by

Volk et al. showing the power of EC/MS in giving insights into biologically complex redox reactions.[44]

The fact that heat is applied to the eluent in the thermospray interface during vaporization and ionization leads to several limitations of EC/MS. The temperature conditions during TSP vaporization change in less than one second from ca. 25°C to greater than 200°C. The elevated temperatures in the TSP interface can affect the reaction rates of intermediates so the reaction pathways are not directly comparable to the reactions at room temperature because the reaction kinetics can be significantlly altered. Thermally labile intermediates and products may also be affected by the elevated temperature of the interface. These compounds can be degraded complicating the interpretation of mass spectral data.

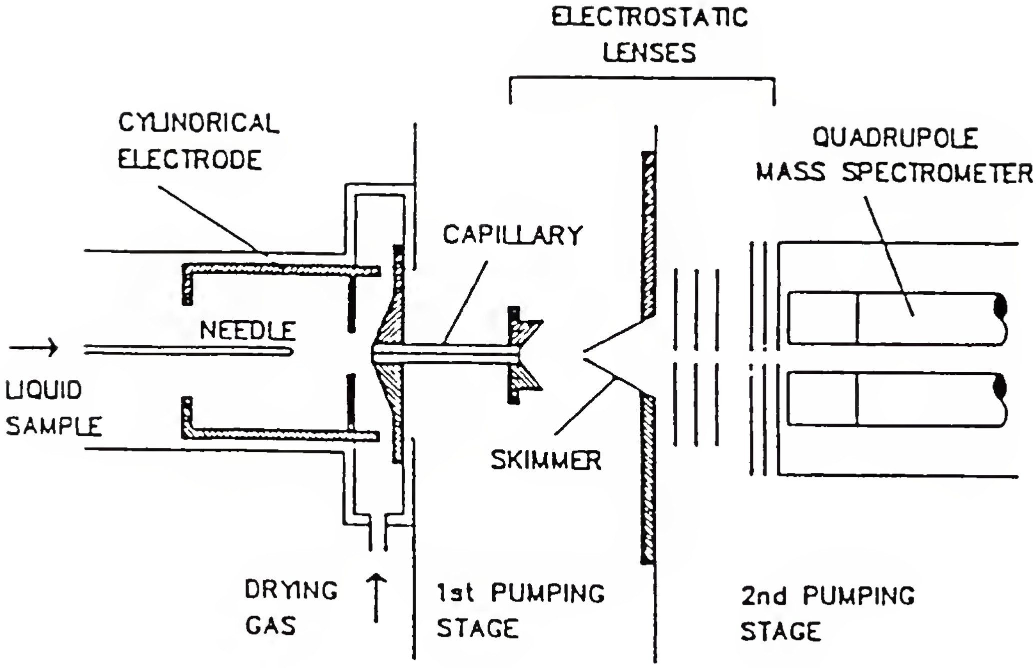
Electrospray Ionization

The evolution of electrospray (ESP) as a viable liquid interface for MS provides a new technique for interfacing EC/MS. The ESP interface heat requirement of ca. 70°C is much lower than that of TSP making ESP more useful for detection of thermally unstable compounds. Vaporization and ionization are accomplished through application of a large potential, typically 2-5 kV. Therefore, the reaction kinetics of the solution analytes should become comparable to the room temperature kinetics, and thermally labile compounds degradation should be slowed down. Another advantage of ESP is its extended mass range of greater than 100,000 daltons. Electrospray-MS has been used to determine accurate molecular weights of proteins with MW over 50,000 and in some cases well above 100,000.[45]

Figure 1.11 shows a diagram of the electrospray interface. The electrospray process requires two steps: dispersal of highly charged droplets at near atmospheric pressure followed by droplet evaporation. Electrospray is produced by the application of a high electric field to a small flow of liquid (typically 1-10μL/min) in a capillary tube. The electric field results in charge accumulation on the liquid surface resulting in the formation of highly charged liquid droplets. After desolvation of these droplets, the ions formed are sampled by the mass spectrometer through a small orifice.[46]

The lower heat requirement and the extended mass range of electrospray makes it a viable interface for EC/MS, as thermally unstable reactants, intermediate and products of electrochemical reactions should be more easily monitored. With the capability of the extended mass range, complex redox reactions of large biological molecules such as proteins and DNA fragments may be investigated by EC/MS.

Figure 1.11: Diagram of Electrospray Interface.[40]


ELECTROSTATIC LENSES
CYLINDRICAL ELECTRODE
QUADRUPOLE MASS SPECTROMETER
CAPILLARY
NEEDLE
LIQUID SAMPLE
SKIMMER
DRYING GAS
1st PUMPING STAGE
2nd PUMPING STAGE

CHAPTER 2

STRUCTURES AND ELECTRON CAPTURE NEGATIVE ION MASS SPECTRA OF FLUORINATED DERIVATIVES OF 21 AMINO ACIDS FORMED BY ONE-STEP ACYLATION/ESTERIFICATION REACTIONS

Introduction

Amino acid analysis is important in biological science with the profiling of free amino acids finding broad clinical applications especially in disease diagnosis.[3] Gas Chromatography/Mass Spectrometry (GC/MS) analysis of amino acids has generated a lot of interest because of the analytical power of MS.[6] Mass spectrometry has the potential to provide definitive qualitative and quantitative information about many organic molecules.[3] In combination with GC, MS provides the sensitivity and selectivity needed for the separation and detection of amino acids in complex matrices, such as bodily fluids, with limited sample clean-up.[4] HPLC is the most popular technique for analyzing amino acids. Unusual non- protein amino acids are often difficult to identify by HPLC as sensitivity is often a problem.

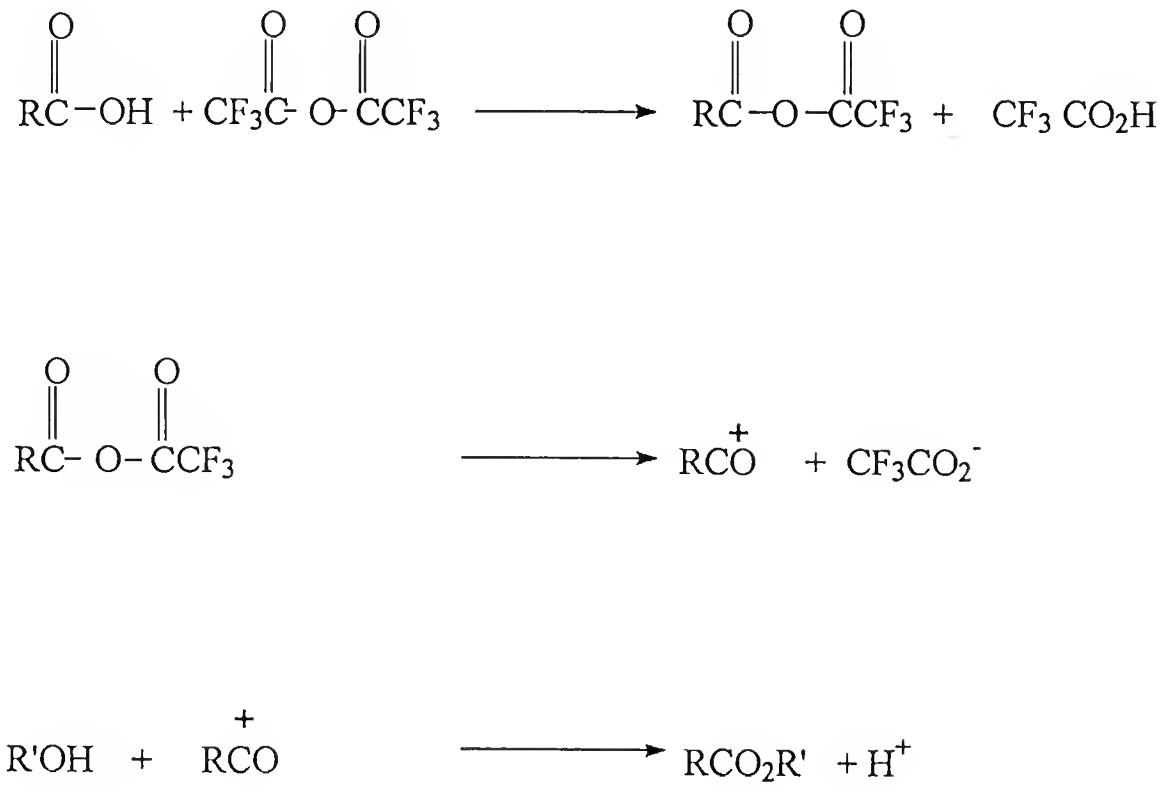
As has been described in Chapter 1 trimethylsilyl (TMS) derivatives and the N-perfluoroacyl alkyl esters (TAB amino acids) are the most important derivatives that allow qualitative and quantitative analysis of amino acids.[5] However the ease of hydrolysis of TMS groups makes them much less suitable for GC/MS analysis than the TAB amino acids. Amino acid derivatization with TAB involves a two-step synthesis in which the amino acid's carboxyl groups are esterified with n-butanol and the amino and hydroxyl groups of the resulting ester are acylated with trifluoroacetic anhydride (TFAA) producing N-TFA n-butyl esters.[5]

The high sensitivity and selectivity of electron capture negative ion (ECNI) mass spectrometry can be exploited for amino acid analysis with the introduction of electrophilic groups as in N-TFA n-butyl esters. For ECNI MS analysis the analyte must have a positive electron affinity and a large cross section for electron capture. These conditions are met in N-TFA n-butyl esters because of the presence of the fluorine atoms which are very electronegative.[19]

It is known that trifluoroacetic anhydride TFAA and its homologs promote rapid esterification of organic and inorganic oxyacids by alcohols via a three-step pathway [13,14] (Figure 2.1). The component responsible for this rapid esterification is the unsymmetrical anhydride (reaction 1), which dissociates to form the acylium cation and the trifluroacetate ion (reaction 2). Even though the concentration of the acylium cation is small its reactivity is sufficiently fast so that rapid esterification from the nucleophilic attack by the alcohol can still occur (reaction 3).

This catalytic property of TFAA, maybe used for simultaneous acylation/esterification of polyfunctional compounds. TFAA or its higher homologs can acylate the compounds, while esterification is by the reaction with an alcohol such as trifluoroethanol (TFE), or its higher homologs. TFAA acylates the primary amine group of amino acids and catalyzes the esterification of the oxyacid by an alcohol. A fluorinated alcohol such as trifluoroethanol (TFE) or pentafluoropropanol (PFP) produces electrophilic

Figure 2.1 Pathway for Catalytic Esterification of Oxyacids by TFAA.



$$\mathrm{R\overset{\overset{\displaystyle O}{\|}}{C}{-}OH} + \mathrm{CF_3\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}CF_3} \longrightarrow \mathrm{R\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}CF_3} + \mathrm{CF_3\,CO_2H} \tag{1}$$

$$\mathrm{R\overset{\overset{\displaystyle O}{\|}}{C}{-}O{-}\overset{\overset{\displaystyle O}{\|}}{C}CF_3} \longrightarrow \mathrm{R\overset{+}{C}O} + \mathrm{CF_3CO_2^-} \tag{2}$$

$$\mathrm{R'OH} + \mathrm{R\overset{+}{C}O} \longrightarrow \mathrm{RCO_2R'} + \mathrm{H^+} \tag{3}$$

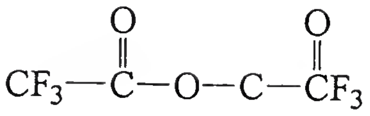
derivatives that are highly sensitive to detection by ECNI-MS. These reactions have been exploited to produce analytically useful derivatives of several polyfunctional molecules.[47,48,15]

In this study, the applicability of simultaneous derivatization with TFAA and TFE for the GC/MS analysis of amino acids using ECNI detection is explored. The one step formation of TFAA/TFE derivatives is an advantage over other techniques previously used, where acylation and esterification was carried out in two separate steps. Single-step acylation/esterification was used to obtain the amino acid derivative, para-aminobenzoyl glutamic acid (pABG), an end product of the metabolism of folic acids in humans.[15] In this study TFAA promotes the esterification reaction of the alpha carboxyl group by TFE and also an internal cyclization reaction. This stabilizes the derivative's molecular anion producing a single ion for ECNI detection. The cyclic derivative provides a sensitive and selective ion that may be detected by selected ion monitoring (SIM). This, along with the time and material conserved, are the major advantages of the single-step acylation/esterification derivatization.

In this chapter the results of simultaneous acylation and esterification of the 20 'standard' amino acids, and cystine, using three combinations of fluorinated reagents will be reported and discussed. The reagent combinations used are: 1) TFAA/TFE, 2) TFAA/PFP, 3) PFPA/ TFE. Structures of the reagents are shown in Figure 2.2.

In the following description the 20 standard amino acids and cystine are classified into the following six different categories according to their side chains.

Figure 2.2 Flourinated Reagents

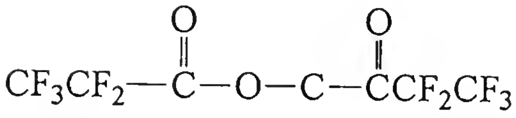


$$CF_3-\overset{\overset{\displaystyle O}{\|}}{C}-O-\overset{\overset{\displaystyle O}{\|}}{C}-CF_3$$

TRIFLUOROACETIC ANHYDRIDE (TFE)

CF_3CH_2-OH

TRIFLUROETHANOL (TFE)



$$CF_3CF_2-\overset{\overset{\displaystyle O}{\|}}{C}-O-\overset{\overset{\displaystyle O}{\|}}{C}-CCF_2CF_3$$

PENTAFLUOROPROPIONIC ANHYDRIDE (PFP)

$CF_3CF_2CH_2-OH$

PENTAFLUOROPROPANOL (PFP)

Amino Acids With Hydrocarbon Side Chains

The structures of the six amino acids with hydrocarbon side chains are shown in Figures 2.3a and 2.3b. Glycine, alanine, valine, leucine and isoleucine have aliphatic side chains ranging from an H atom in glycine to an iso-butyl group for leucine and isoleucine. Phenylalanine is aromatic and has a phenyl moiety. Proline (Figure 2.3b) which has a secondary amine and is actually a cyclic α-imino acid is commonly referred to as an amino acid and can also be classified in this group. The side chains in this group of amino acids are expected to be unreactive during the derivatization procedure, therefore, only acylation of the amine group and esterification of the acid is expected.

Amino Acids with Alcoholic Side Chains

Three amino acids serine, threonine and tyrosine have side chains with alcoholic groups. Their structures are shown in Figure 2.4. Serine and threonine have hydroxyl side chains of different sizes while tyrosine has a phenolic side chain. The hydroxy groups are expected to undergo acylation by the anhydride during derivatization.

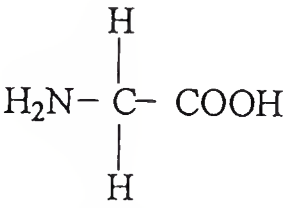
Amino Acids with Acidic Side Chains

The structures of the two acidic amino acids, aspartic acid and glutamic acid are shown in Figure 2.5. They are both dicarboxylic acids which makes ring closure possible during derivatization.

Amino Acids with Amide Side Chains

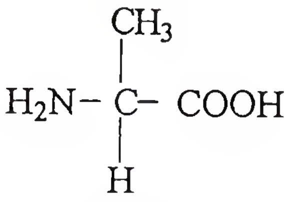
Asparagine and glutamine (Figure 2.6) are amides of aspartic acid and glutamic acid respectively. Their reactivity is expected to be similar to that of the dicarboxylic acids, although the unreactivity of certain amides under these conditions has been reported.[48]

Figure 2.3a Structure of Amino Acids with Hydrocarbon Side Chains.



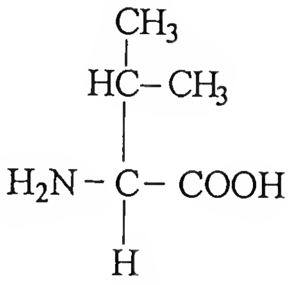
$\mathrm{H_2N-\overset{\displaystyle H}{\underset{\displaystyle H}{C}}-COOH}$

Glycine
(gly)

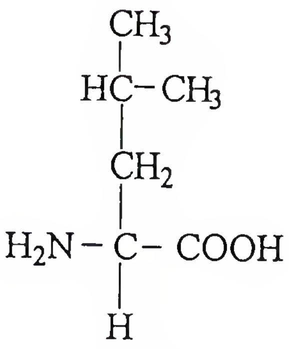


$\mathrm{H_2N-\overset{\displaystyle CH_3}{\underset{\displaystyle H}{C}}-COOH}$

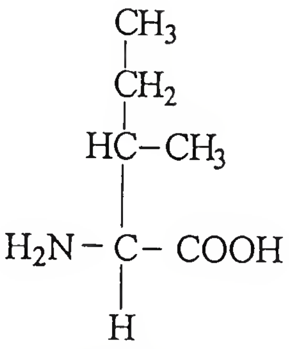
Alanine
(ala)



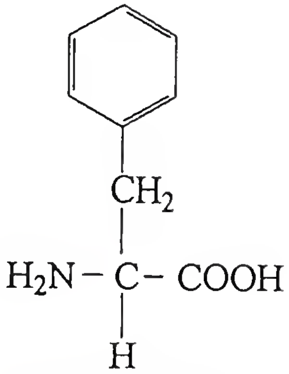
Valine
(val)



Leucine
(leu)

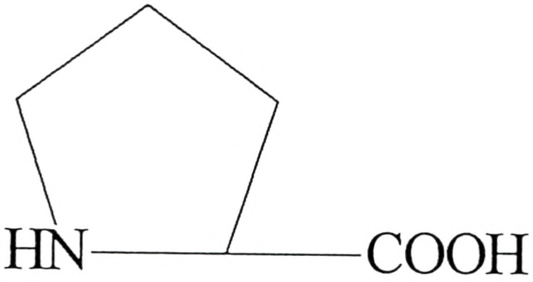


Isoleucine
(ile)



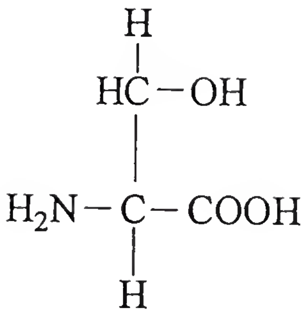
Phenylalanine
(phe)

Figure 2.3 b Structure of a Cyclic Imino Acid, Proline

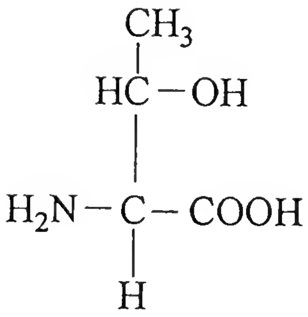


Proline
(pro)

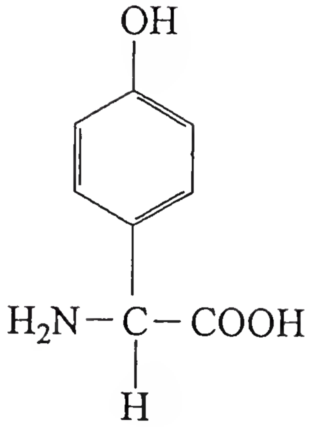
Figure 2.4 Structures of Amino Acids with Alcoholic Side Chains.



Serine
(ser)

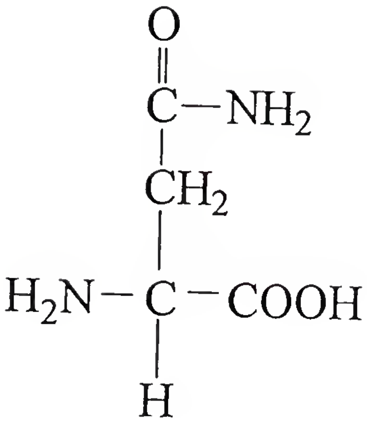


Threonine
(thr)

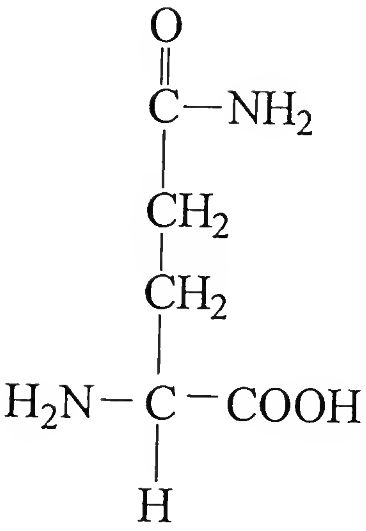


Tyrosine
(tyr)

Figure 2.5 Structures of Amino Acids with Acid Side Chains.

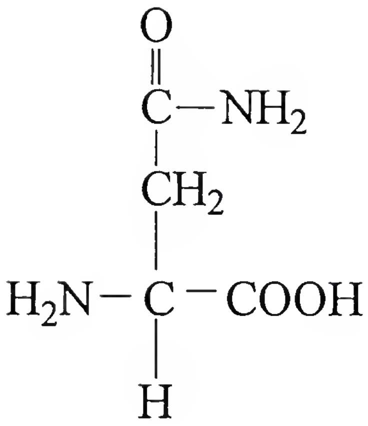


Aspartic Acid
(asp)

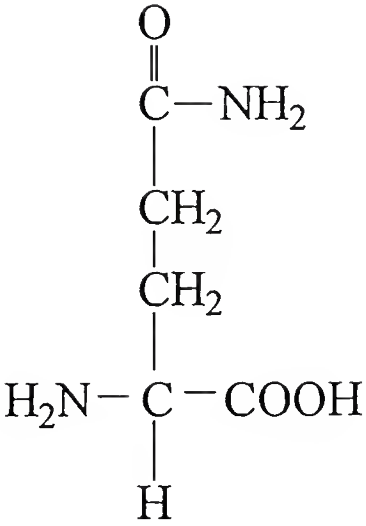


Glutamic Acid
(glu)

Figure 2.6 Structures of Amino Acids with Amide Side Chains



Asparagine
(asn)



Glutamine
(gln)

Amino Acids with Basic Side Chains

Figure 2.7 shows the structure of the four amino acids which are classified as having basic side chains: lysine, which has butylammonium group; arginine, which bears a guanidino group; histidine, which carries an imidazolium side chain and tryptophan, which has an indole group as its side chain. The basic side chains may undergo acylation by the anhydride at the amine group during derivatization.

Amino Acids with Sulfur Containing Side Chains

Cysteine and methionine are amino acids with sulfur containing side chains (Figure 2.8). Cysteine has a thiol group and is unique among the ' standard' amino acids because it can form a disulfide bind with another cysteine to make the dimer, cystine. Methionine has a thiol ether side chain. The sulfur containing side chain should be acylated at the thiol group during derivatization.

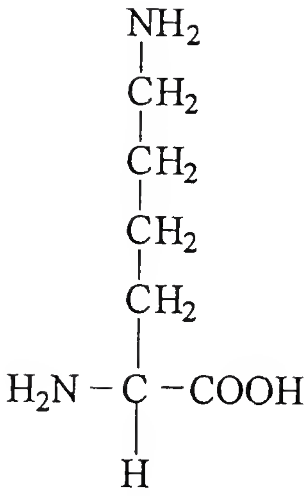
Experimental

Reagents and Materials

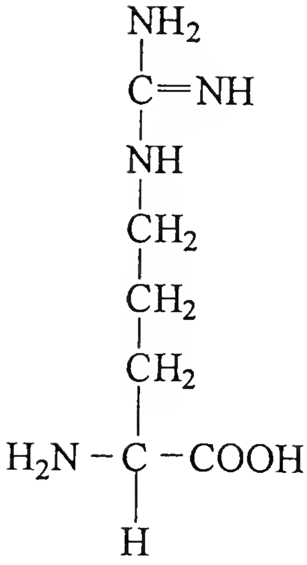
Amino acid standards were obtained from Sigma Chemicals. Trifluoroacetic anhydride (TFE), pentafluoropropionic anhydride (PFPA), trifluoroethanol (TFE) and pentafluoropropanol (PFP) were obtained from PCR Chemicals. Other reagents and solvents were obtained from Fisher Scientific. Ethyl acetate and the fluorinated alcohols were stored over molecular sieves. All other materials were used as received.

Stock solutions of amino acids were prepared by dissolving accurately weighed samples of amino acids in 0.1 N HCl. All stock solution concentrations ranged from 2-8 μM/mL.

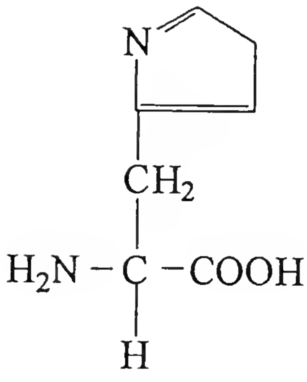
Figure 2.7 Structures of Amino Acids with Basic Side Chains.



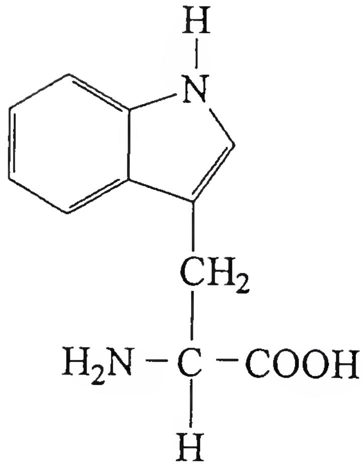
Lysine
(lys)



Arginine
(arg)

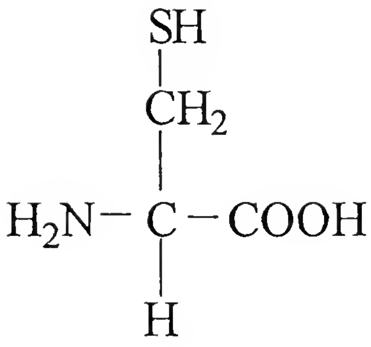


Histidine
(his)



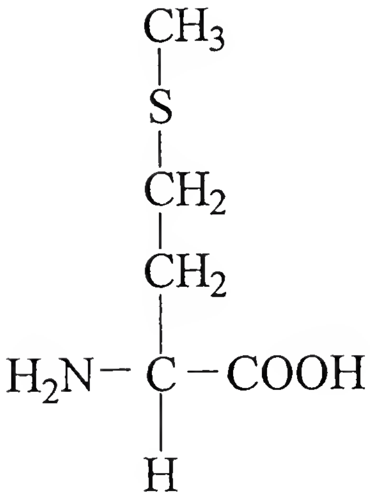
Tryptophan
(trp)

Figure 2.8 Structure of Amino Acids with Sulfur Containing Side Chains.



$$\begin{array}{rcl} & SH & \\ & | & \\ & CH_2 & \\ & | & \\ H_2N- & C & -COOH \\ & | & \\ & H & \end{array}$$

Cysteine
(cys)



$$\begin{array}{rcl} & CH_3 & \\ & | & \\ & S & \\ & | & \\ & CH_2 & \\ & | & \\ & CH_2 & \\ & | & \\ H_2N- & C & -COOH \\ & | & \\ & H & \end{array}$$

Methionine
(met)

Derivatization

Derivatives were prepared by evaporating 50 µL aliquots of amino acid solutions to dryness under a stream of dry nitrogen in glass culture tubes, then adding 200 µL TFAA and 100 µL TFE, tightly capping the tubes, and heating in a metal block for one hour at 80-100°C. After 1 hour, excess reagents were evaporated under dry nitrogen, and the dry residue was dissolved in 100 µL dry ethyl acetate.

Instrumentation

GC/MS data were obtained using a Finnigan Model 4500 GC/MS system (Finnigan MAT, San Jose, CA, USA). Typically, two µL injections representing 2-8 nanomole of the derivative, were made into a 30 m x 0.25 mm DB-17 capillary GC column (J & W Scientific, Folsom, CA, USA) interfaced directly to the mass spectrometer and temperature-programmed from 50°C to 300°C at 12°C/min. Chemical ionization (CI) and electron capture negative ion (ECNI) spectra were obtained using methane as the reagent gas at a pressure of ca. 0.4 Torr.

Derivative structures were inferred from molecular weights obtained from CI data and from observed shifts in molecular weight when PFPA was substituted for TFAA and PFP substituted for TFE. Structures of negative ions in ECNI spectra were elucidated in the same manner.

Results and Discussion

Effect of Derivatizing Reagent on Derivatives

The three combinations of reagents, TFAA/TFE, TFAA/PFP and PFPA/TFE, produced analogous derivatives. The structures of the derivatives were not affected by the combination of reagents. However, there were differences in the ENCI fragmentation mechanisms depending on which of the three anhydrides were used. For about half of the TFAA/TFE and TFAA/PFP derivatives formed, the base peak in the ECNI spectrum is the $[CF_3CONH]^-$ ion, which is unrelated to the structure of the parent amino acid and therefore provides no analytically useful information. The analogous PFPA/TFE derivatives produce an analytically more useful $[M-H]^-$ ion as the base peak. Only the more analytically useful PFPA/TFE derivatives are reported and discussed here. The choice of alcohol had no effect on the ECNI mass spectra.

Negative Ion Chemical Ionization Mass Spectrometry (NICI-MS)

Structures of Derivatives.

Derivatives with Hydrocarbon Side Chains

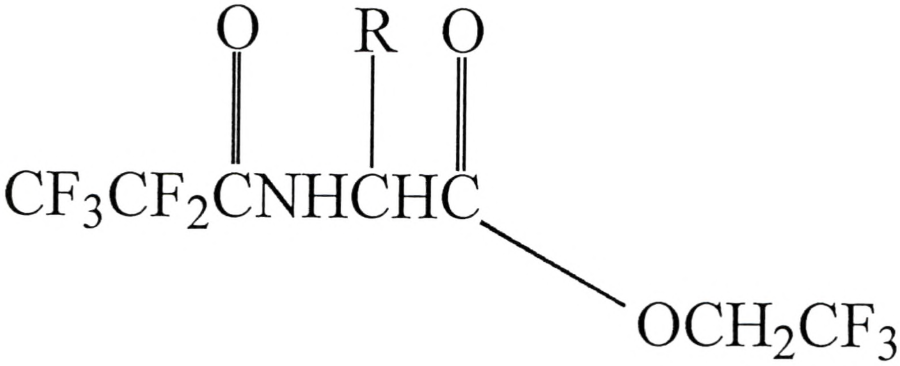
The six amino acids with hydrocarbon side chains: alanine, valine, leucine, isoleucine, glycine and phenylalanine have no reactive functional groups other than the alpha amino and carboxyl groups which undergo the acylation/esterification reaction. A single derivative with the general structure **I** (Figure 2.9) is formed in each case.

Proline forms two products (Figure 2.10). In addition to the expected product of acylation of the amino group and esterification of the imino acid **II**, there is also a second product **III** which is the unsaturated analog of **II**.

Amino Acids with Alcoholic Side Chains

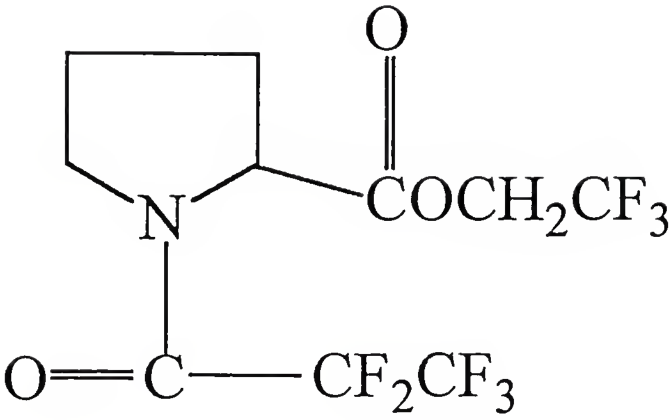
Serine, threonine, and tyrosine are amino acids with alcohol containing side groups. In addition to the expected acylation/esterification reactions the hydroxy group is acylated in all cases. The structure of the serine derivative **IV** is shown in Figure 2.11. Threonine and

Figure 2.9 Structure of PFPA/TFE Derivatives with Unreactive Side Chains

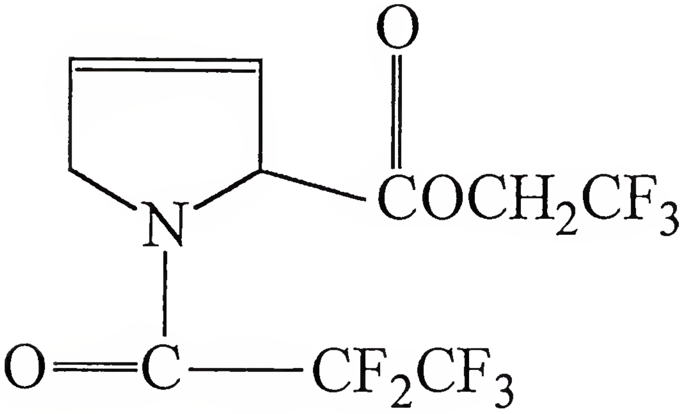


I

Figure 2.10 Sructure of Proline PFPA/TFE Derivatives

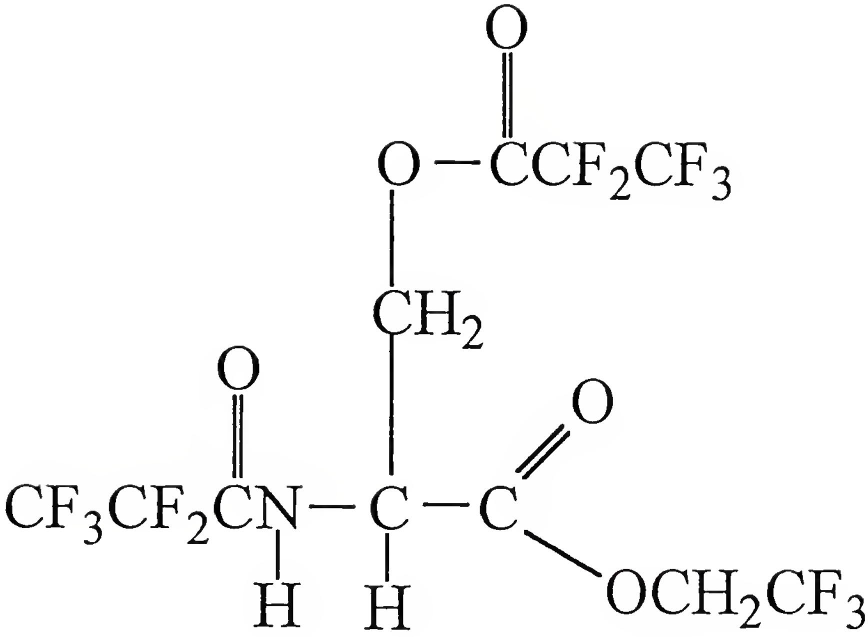


II



III

Figure 2.11 Structure of Serine PFPA/TFE Derivative.



IV

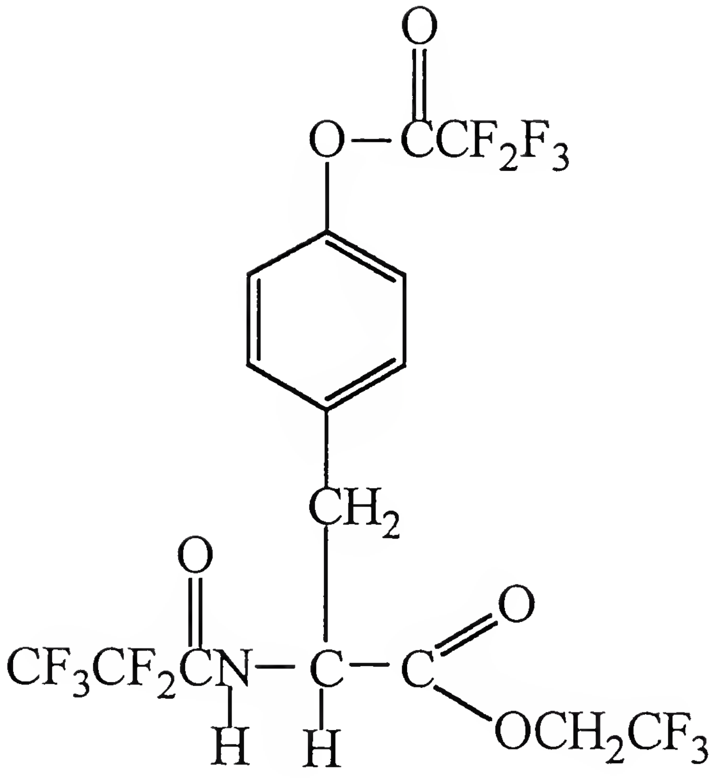
tyrosine form analogous derivatives. Tyrosine forms only a single derivative immediately after preparation. After a few days, a second compound which is consistent with a product with an underivatized phenolic hydroxyl group **V** (Figure 2.12) was identified. It has been reported that trifluoroacyl derivatives of phenolic compounds establish an equilibrium for the hydrolysis reaction after a few days.[47]

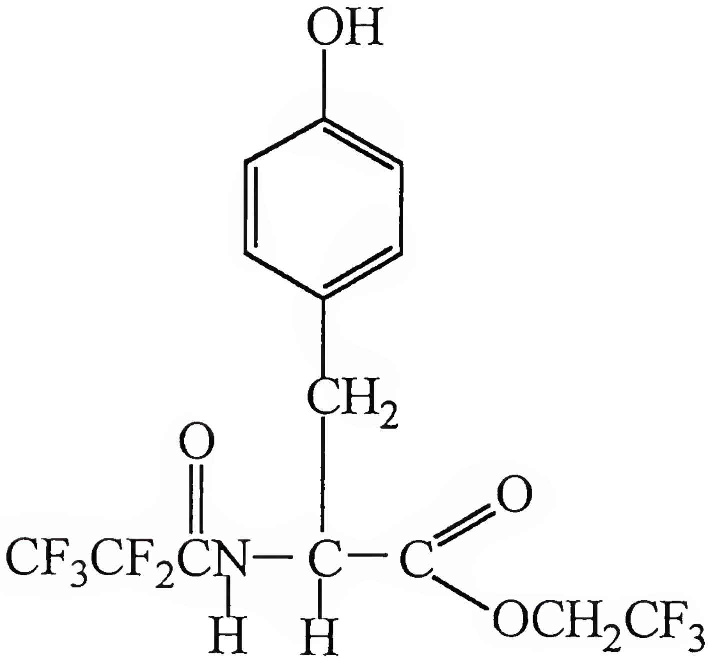
Amino Acids with Acidic Side Chains

The dicarboxylic acids, glutamic acid and aspartic acid, form derivatives with the structure **VI** (Figure 2.13). The derivative is formed from the acylation of the amine group and the esterification of both carboxylic groups by the anhydride and alcohol respectively. Aspartic acid forms a single derivative while glutamic acid forms two major derivatives: one analogous to that formed by aspartic acid and a cyclic derivative **VII** (Figure 2.14).

The cyclic glutamic derivative, **VII**, is consistent with the ring closure reactions observed with pAGB[15] in the formation of a lactam structure. Ring closure is possible during derivatization if the amino acid contains an additional reactive site on its side chain. The proposed pathway for the formation of the glutamic acid lactam derivative is shown in Figure 2.15. This pathway is consistent with that of Bourne et al[13,14] where the anhydride, PFPA, catalyzes the esterification of the carboxyl acids by alcohols. First, there is formation of an asymmetric anhydride **(B)**, which ionizes to a pentafluoroacetate ion **(C)**, and an acylium ion **(D)** which undergoes nucleophilic attack by the alcohol, TFE **(E)**. In the case of glutamic acid, the proximity of the amide nitrogen allows it to compete successfully as a nucleophile with TFE, resulting in an internal nucleophilic derivative **(F)**.

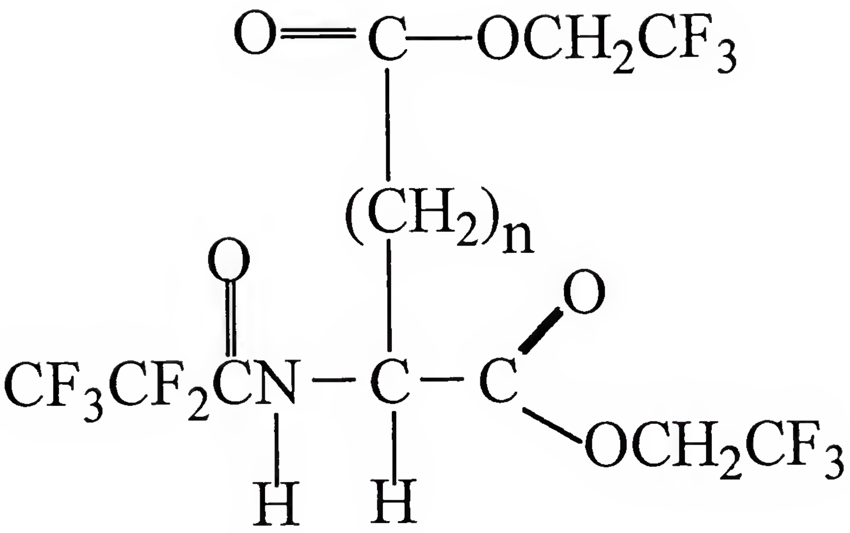
Figure 2.12 Sructure of Tyrosine PFPA/TFE Derivative.





V

Figure 2.13 General Structure of PFPA/TFE Derivatives with Acidic and Amide Side Chains

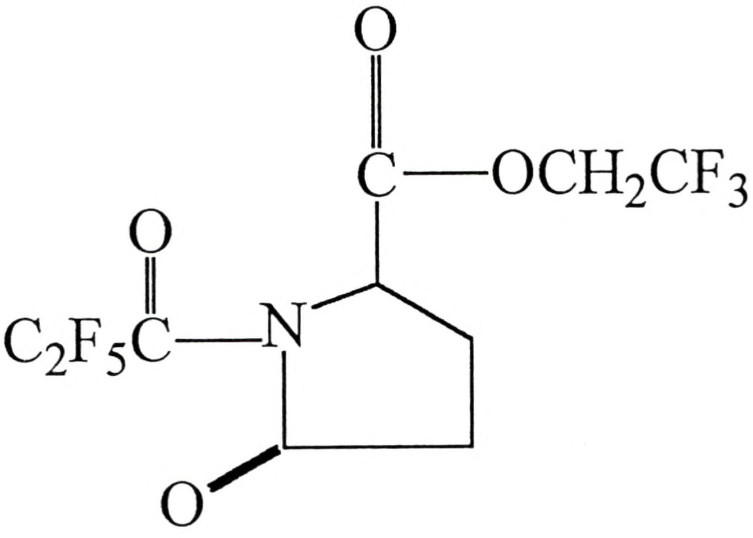


VI

Asp: n = 1

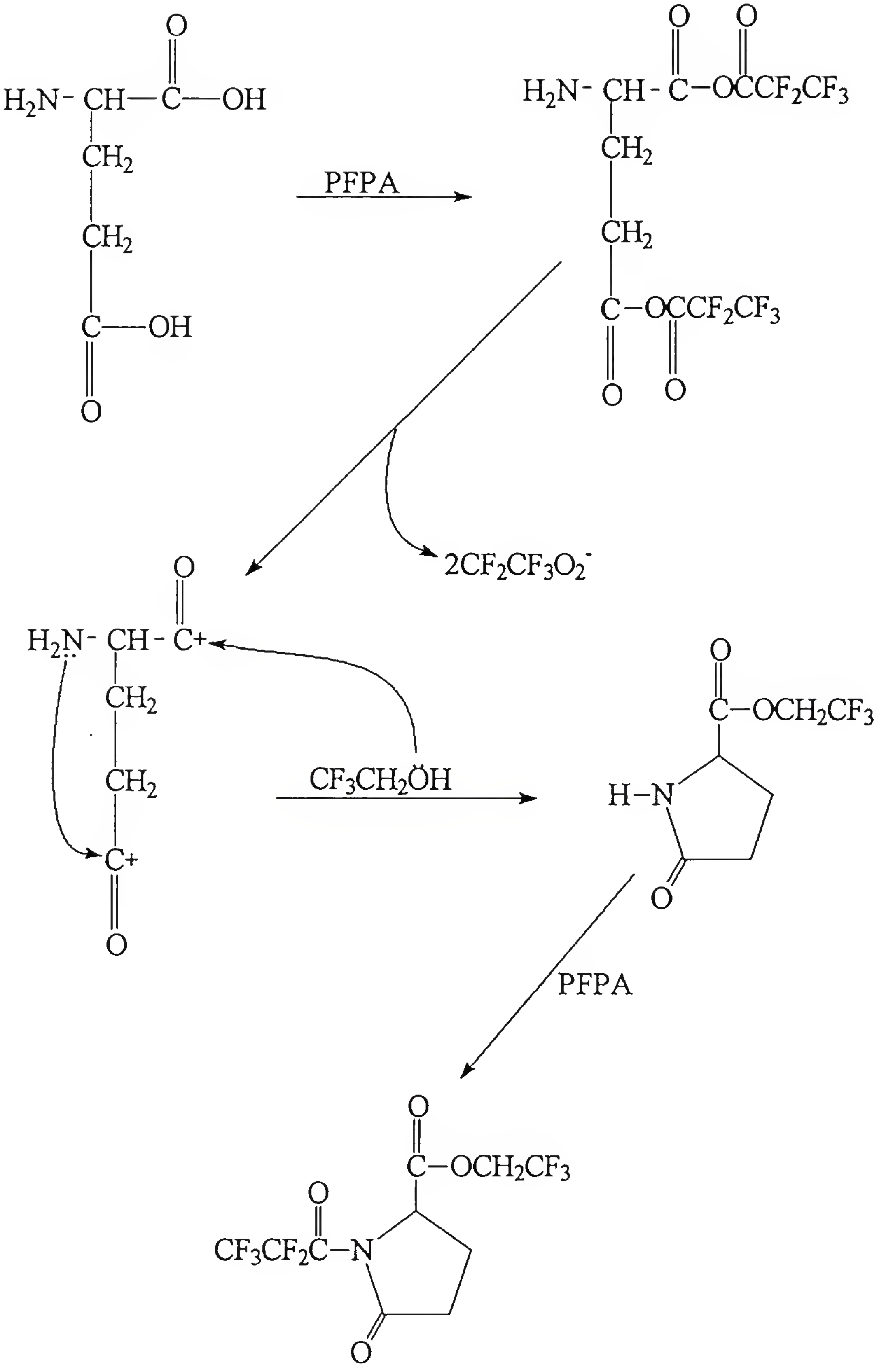
Glu: n = 2

Figure 2.14 Structure of Proposed Cyclic PFPA/TFE Derivative of Glutamic Acid.



VII

Figure 2.15 Proposed Pathway for the Formation of PFPA/TFE Lactam Derivative of Glumatic Acid.


PFPA
2CF2CF3O2-
CF3CH2ÖH
PFPA

Amino Acids with Amide Side Chains

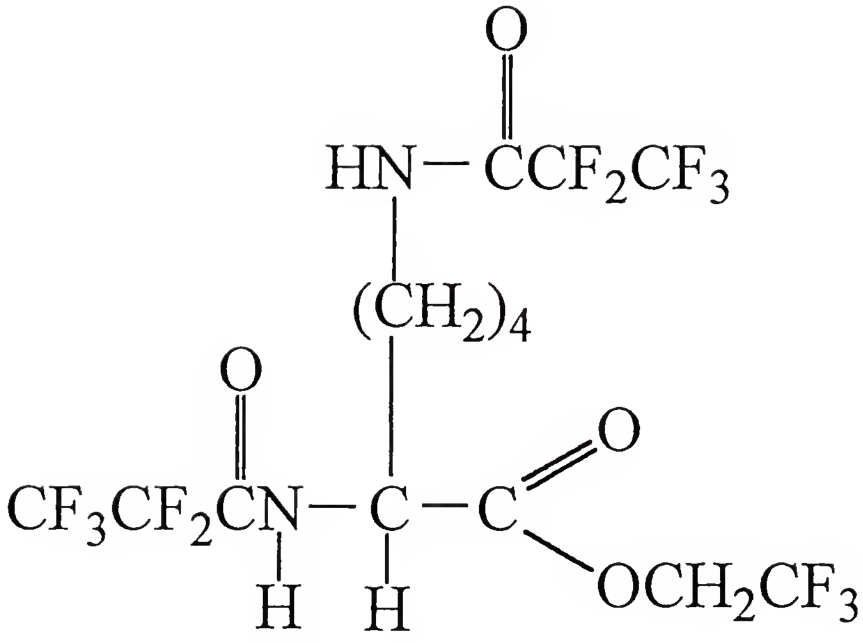
Asparagine and glutamine produced derivatives are identical to those formed by aspartic acid and glutamic acids respectively (Figures 2.13 and 2.14). This suggests that under the reaction conditions used the amide groups of asparagine and glutamine are converted to their acids and subsequently derivatized to the products mentioned above. The difficulty in obtaining derivatives of amides has been discussed previously.[47]

Amino Acids with Basic Side Chains.

Lysine, tryptophan, arginine and histidine are amino acids with basic side chains. The imidazole group of the histidine is unreactive and, therefore, forms the simple derivative **I** as previously discussed for amino acids with hydrocarbon side chains. Lysine forms a single derivative in which the amino group of the side chain is acylated by the anhydride **VIII** (Figure 2.16) .

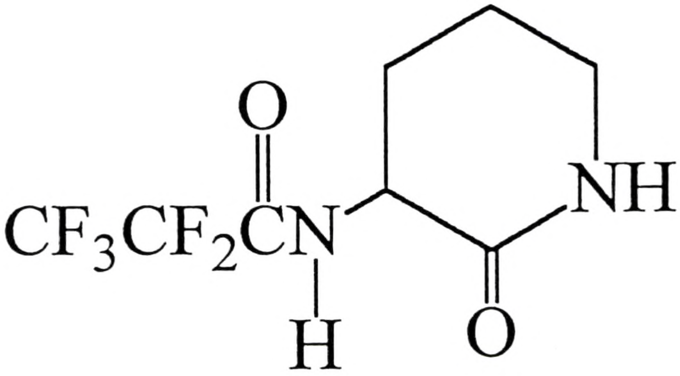
Arginine forms one major product with a lactam structure **IX** (Figure 2.17). The proposed pathway for the formation of the this derivative is similar to that described above for the lactam derivative of glutamic acid (Figure 2.15). The proposed mechanism for the formation of this derivative is similar to that described above for glutamic acid. In ECNI there is evidence of derivatives of ornithine **X, XI** (Figure 2.18) being formed, which elutes slightly earlier than the lactam derivative with about 50% of the intensity of the lactam derivative. Ornithine **XII** (Figure 2.19) is an unessential amino acid which is an intermediate in the *in vivo* formation of arginine.[49] This compound is not detected by EI or PCI. The formation of t-butyldimethylsilyl (tBDMS) ornithine has been reported during the preparation of tBDMS derivatives of arginine.[50]

Figure 2.16 Structure of Lysine PFPA/TFE Derivative.



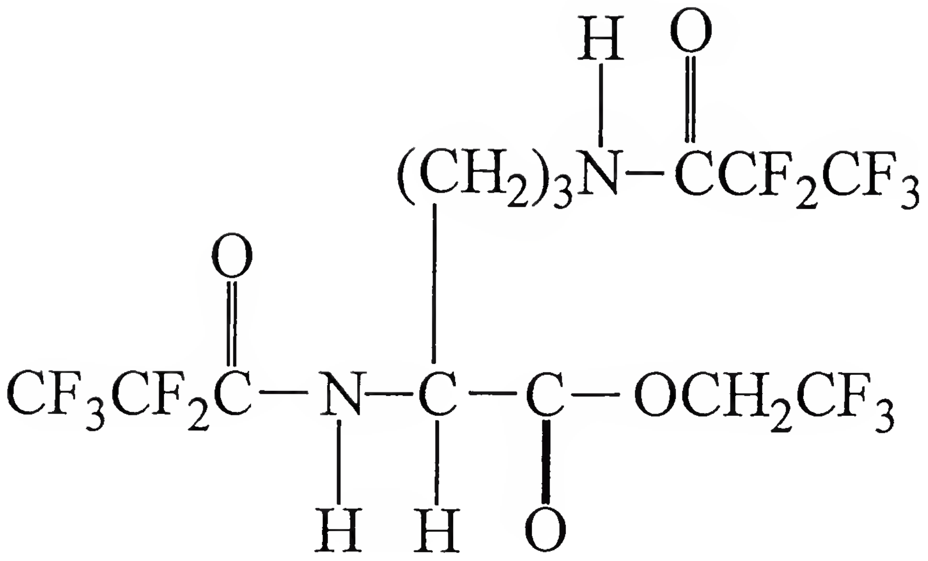
VIII

Figure 2.17 Structure of Arginine PFPA/TFE Derivative.

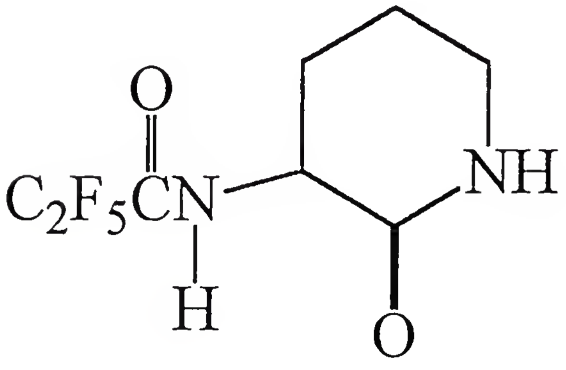


IX

Figure 2.18 Structure of Ornithine PFPA/TFE Derivative.

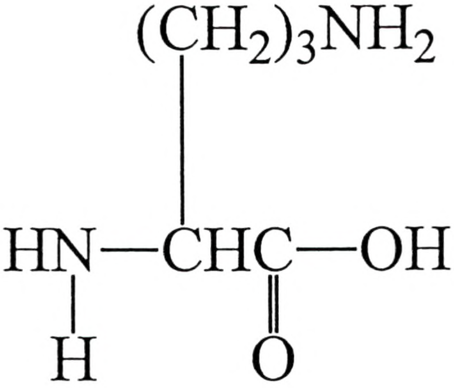


X



XI

Figure 2.19 Structure of the Amino Acid Ornithine



$\mathrm{HN(H)\!-\!CH[(CH_2)_3NH_2]C(=O)\!-\!OH}$

XII

Tryptophan forms two major products. In one form the nitrogen of the indole group is acylated by the anhydride **XIV** (Figure 2.20) as reported by Duffield et al.[21] and in the other form, the indole group is underivatized **XIII**. This is analogous to the equilibrium set-up in the case of tyrosine. The sensitivity of detection of the fully derivatized species **XIV** compared to the partially derivatized species **XII** is much greater, even though the concentrations may be equal. This due to the presence of an additional C_2F_5 site in **XIV** which results in a larger electron capture cross section .

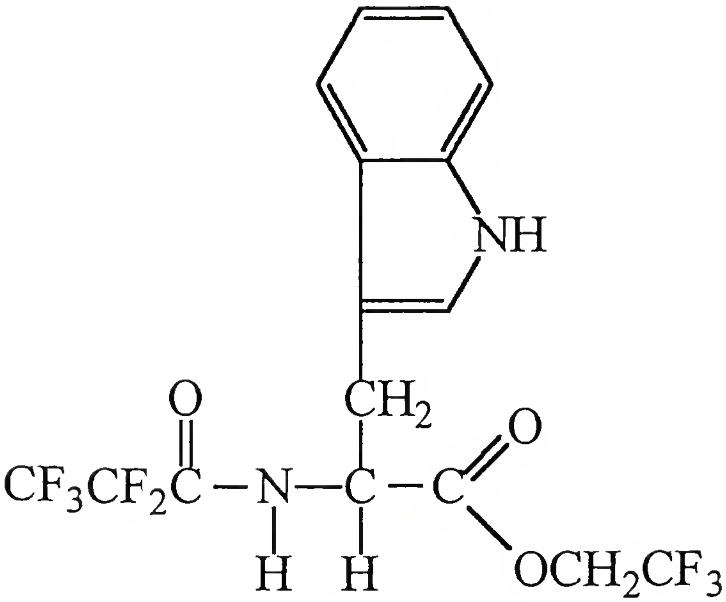
Amino Acids with Sulfur Containing Side Chains

Methionine, cysteine and cystine are the three amino acids with sulfur containing side chains. The methylmercapto side chain of methionine is unreactive and a simple derivative, analogous to those of the aliphatic amino acids **I**, is formed. Cysteine forms a major product with the structure **XIV** (Figure 2.21) where in addition to the expected acylation/esterification there is also acylation of the thiol group by the anhydride. Cystine forms a derivative identical to the cysteine derivative suggesting that under the reaction conditions cystine is cleaved to cysteine prior to derivatization. Cysteine also forms several low concentration minor products with high sensitivity to ECNI detection. The structures of these compounds cannot be inferred from their ECNI spectra.

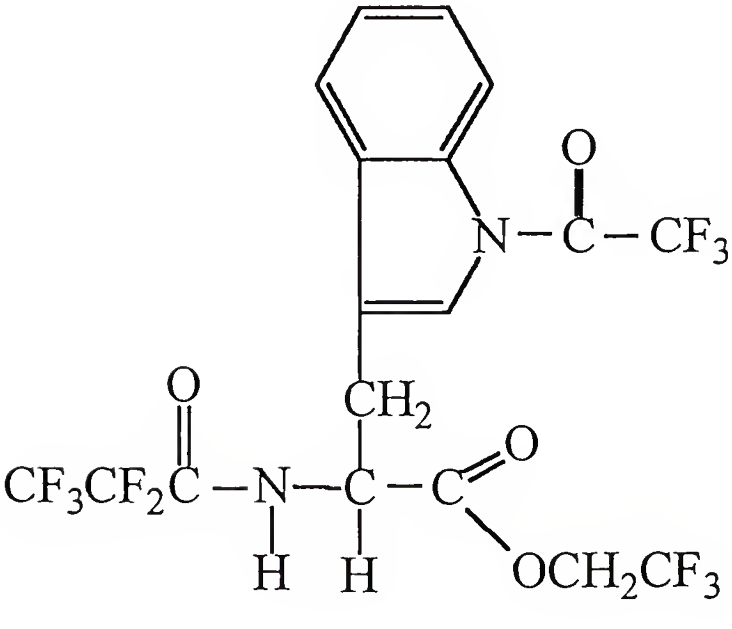
Conclusions

In this chapter the feasibility of simultaneous acylation and esterification of 21 amino acids using perfluorinated acid anhydrides and fluorinated alcohols was investigated. For 15 of the amino acids investigated the advantages in detection of derivatives formed by this

Figure 2.20 Structures of Tryptophan PFPA/TFE Derivatives.

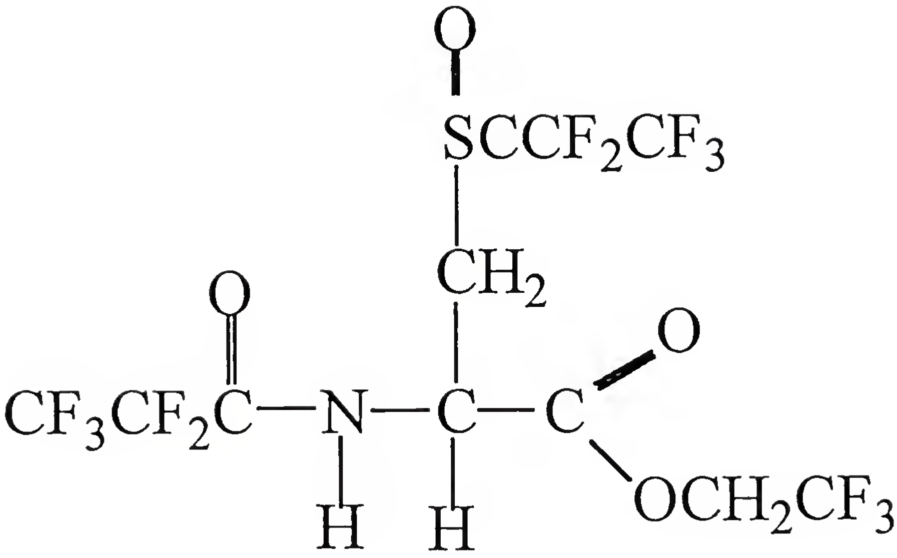


XIII



XIV

Figure 2.21 Structure of Cystine PFPA/TFE Deivatives



XV

method equal or exceed those of PFP n-butyl derivatives with the additional advantage of a single reaction step. In each case ECNI produces a base-peak ion characteristic of the amino acid, which makes it therefore suitable for detection by selected ion monitoring (SIM).

The nine amino acids with unreactive side chains, along with serine and threonine, form derivatives equal to ENCI detection to their N-PFP n-butyl counterparts. Aspartic acid and lysine form derivatives that have $[M-HF]^-$ as the base peak. Tyrosine forms two derivatives which could be a disadvantage from an analytical standpoint. However the partially derivatized product produces a diagnostic $[M-HF]^-$ base peak in the ECNI spectrum while its fully derivatized analogue does not. This partial derivative is also superior to the PFP-n butyl derivatives. Arginine forms two products detectable by ECNI which can be separated by GC, which appears not to be the case for N- PFP n-butyl derivatives. The lactam derivative, the major product, produces an analytically useful ECNI spectrum.

The remaining six amino acids are incompatible for use with the single step derivatization method. Asparagine, glutamine and cystine produce contradictory derivatives, as the amino acids are converted to aspartic acid, glutamic acid and cysteine respectively during the derivatization reaction. Glutamic acid, tryptophan and cysteine form multiple ECNI sensitive products, which limits the use a single step acylation/esterification for analysis of these amino acids.

The single step acylation/esterification is impractical for total amino acid analysis of protein hydrolysates, but can provide rapid analysis and high sensitivity for the detection of specific amino acids.

CHAPTER 3
ELECTROCHEMISTRY OF MITOMYCIN C

Introduction

As previously mentioned in Chapter 1, mitomycin C (MC) is a quinone antitumor antibiotic that covalently binds to cellular nucleotides such as DNA,[51] RNA[52] and proteins,[53] and crosslinks complimentary DNA strands.[54] The covalent attachment of MC to DNA accounts for its cancer fighting ability. The anticancer activity of MC is contingent upon its reduction by intracellular enzymes.[55] The major bioreductive catalysts for MC reductive activation are cytochrome P-450 reductase and xanthine oxidase which are membrane bound flavoenzymes.[56,57] A variety of reductants including, enzymatic,[27] catalytic,[27] chemical,[25,27] electrochemical[26,32,33,35] and radiolytic,[58] have been used to activate MC *in vitro* to provide information about the mechanism of the drug action . Despite the convincing evidence for the need of bioreductive activation of MC there is an enigma as to the role of the one-electron versus two electron reduction of the quinone moiety in the bioactivity of the drug. Most of the earlier proposed mechanisms for the drug activity of MC assumed the two electron reduction of MC to the hydroquinone.[59,27,32] The ability of the one-electron reduced radical anion of MC to alkylate DNA was reported by Tomasz et. al..[60] Sodium dithionite was used to model the bioreduction and it was suggested that the primary reactive species binding to the DNA was the radical anion of MC.

Previous Studies of the Electrochemistry of Mitomycin C

The lipid bilayers of cellular membranes are heterogenous consisting of both hydrophilic and hydrophobic regions. The reduction of MC may conceivably take place in the lipid matrix where the radical anion may have a considerable lifetime since cytochrome P-450 is a membrane bound enzyme. The aprotic, lipid environment favors mechanistic pathways different from those in protic environments. So, the study of MC reduction in aprotic organic solvents is relevant to the study of both the metabolic activation and the mechanism of action of MC. Electrochemistry has been used to model the bioreduction of MC in aqueous and in methanolic solutions at hanging mercury drop[28] and platinum electrodes,[32] and in dimethylformamide (DMF) and dimethyl sulfoxide (Me_2SO) at platinum electrodes.[26] The electrochemical reductions of MC in solvents such as DMF, Me_2SO and methanol have been shown to involve the initial formation of an anion radical.[33]

Electrochemistry in Aqueous Solutions

The electrochemical reduction of MC at hanging mercury drop electrodes in buffered aqueous solutions (phosphate buffer, pH 6.98) has been reported by Rao et al..[32,61] In the cyclic voltammetric data two successive electrochemical reduction peaks were observed at -368 and -468 mV respectively versus SCE at scan rates of 1 V/s. The peaks were attributed to the two-electron, two-proton quinone-hydroquinone reduction of MC (Figure 3.1) and to the two-electron two-proton reduction of the ring-opened hydroxy substituted moiety of MC, 1-hydroxy-2,7-diaminomitosene **4** (Figure 3.2) respectively. It was proposed that the three -membered aziridine ring-opening occurred from the hydroquinone in aqueous media and the quinone radical intermediate was not detected as is generally the case in aqueous media.

Figure 3.1: Two-electron, Two-Proton Reduction of Mitomycin C.

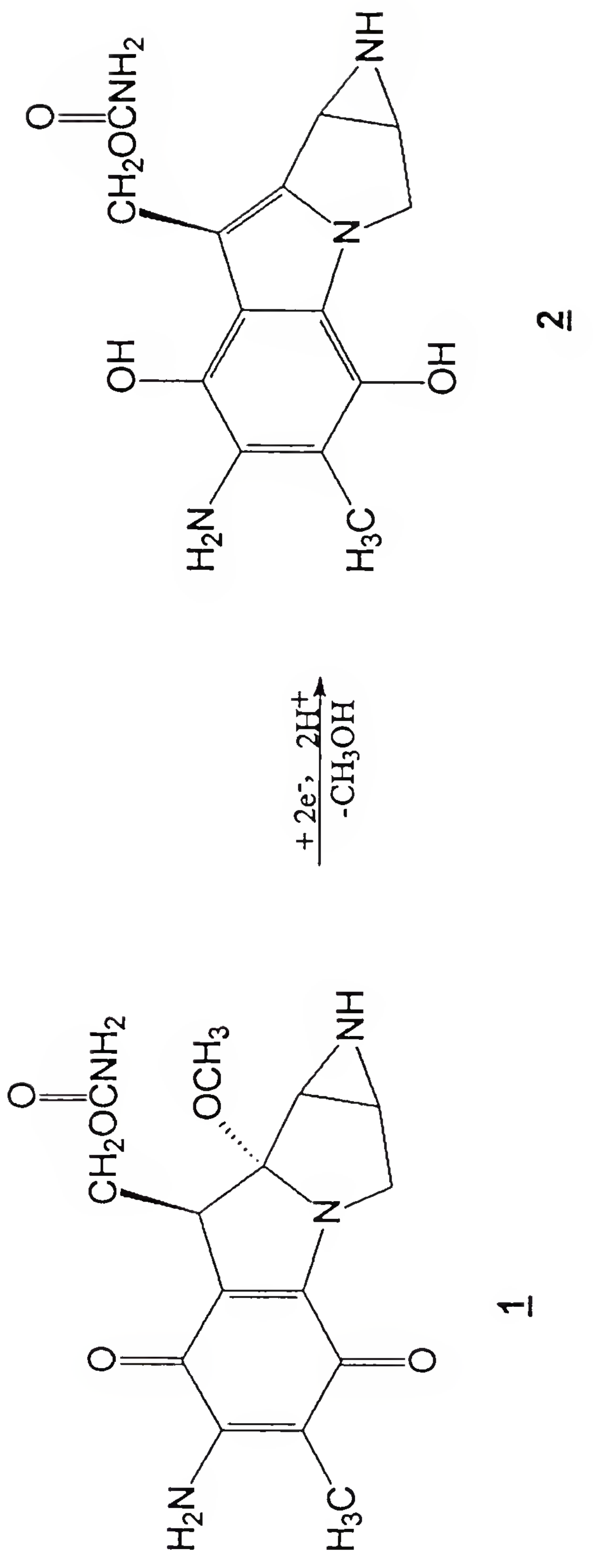
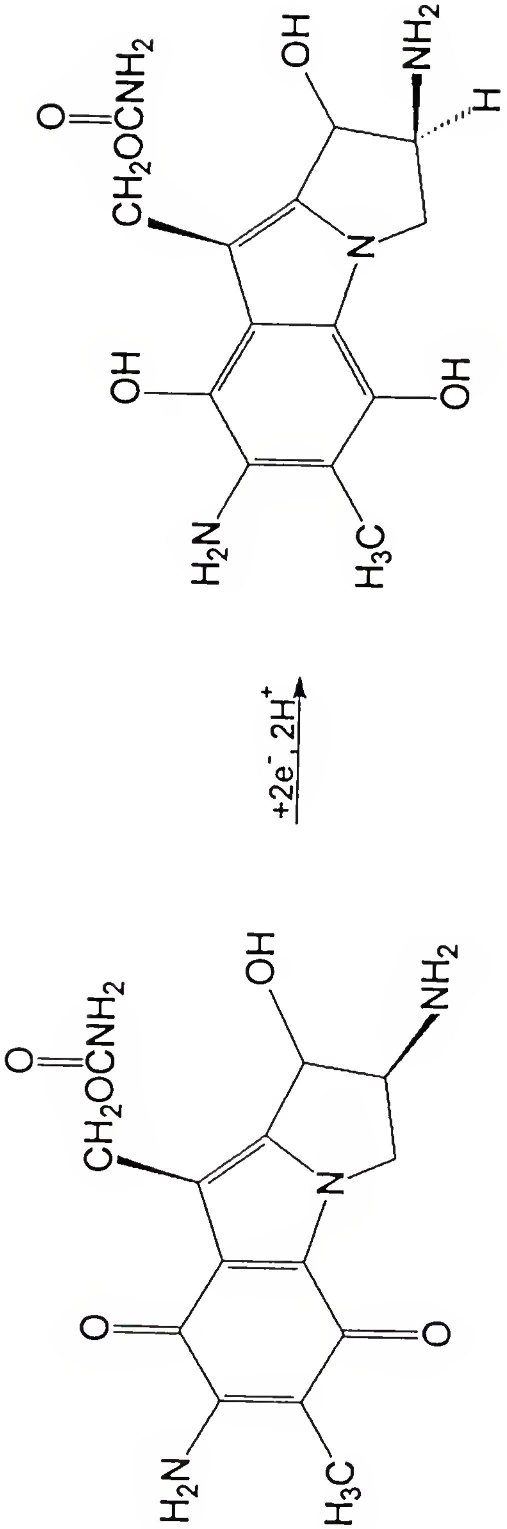

1
2
H2N
H3C
CH2OCNH2
OCH3
NH
N
OH
+ 2e-, 2H+
-CH3OH

Figure 3.2: Two-electron, Two-Proton Reduction of 1-Hydroxy-2,7-diaminomitosene.



4

5

Figure 3.3 shows the proposed mechanism for the reduction of MC in aqueous solutions. According to this mechanism MC **1**, undergoes a two-electron two-proton reduction to the hydroquinone **2**, then there is a loss of the methoxy group as methanol and opening of the strained three-membered arizidine ring forming **3**. The C-10 position of MC is a nucleophilic site where OH^- can attack forming **5**. It was suggested that at the reduction potential of MC, **5** is oxidized to the quinone **4**, which is then present and available for reduction at a more negative potential (Figure 3.2).

Electrochemistry of Mitomycin C in Non-Aqueous Solutions.

To investigate the one-electron reduction of MC to a radical anion, Andrews and co-workers studied the electrochemical reduction of MC in DMF and Me_2SO.[26] Platinum electrodes were used in the studies. The MC radical anion formed from the electrochemical reduction was reported to be stable in the aprotic, anaerobic DMF and Me_2SO solutions. It was concluded that the one-electron reduction was sufficient to activate MC to its biologically active intermediate. The radical formation was suggested as the primary mode of the bioreductive activation of MC,[26] because when water was added following the formation of the radical anion hydrolysis of the radical anion occured. The products generated form the hydrolysis closely resembled the profile of products of the *in vitro* reduction of MC with cytochrome P-450 reductase.

The electrochemical reduction of MC by cyclic voltammetry at mercury and platinum electrodes was investigated by Kohn and coworkers [28] to determine the probable reduction pathway in the protic non-aqueous solvent, methanol. Figure 3.4 shows the proposed mechanism for the reduction of MC in methanol. It was determined [28] that the activation of

Figure 3.3: Proposed Mechanism for the Redction of Mitomycin C in Aqueous Solutions.

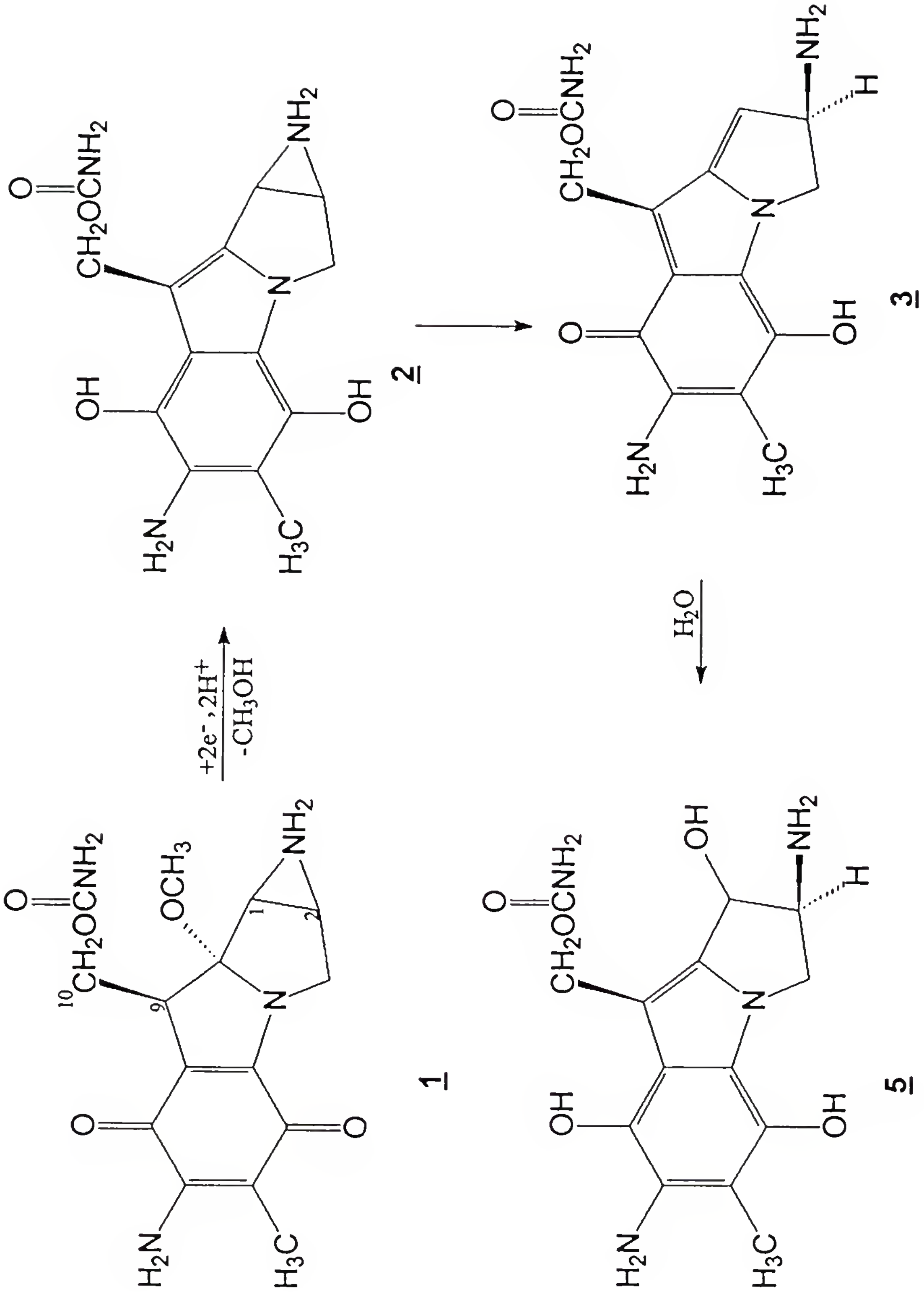
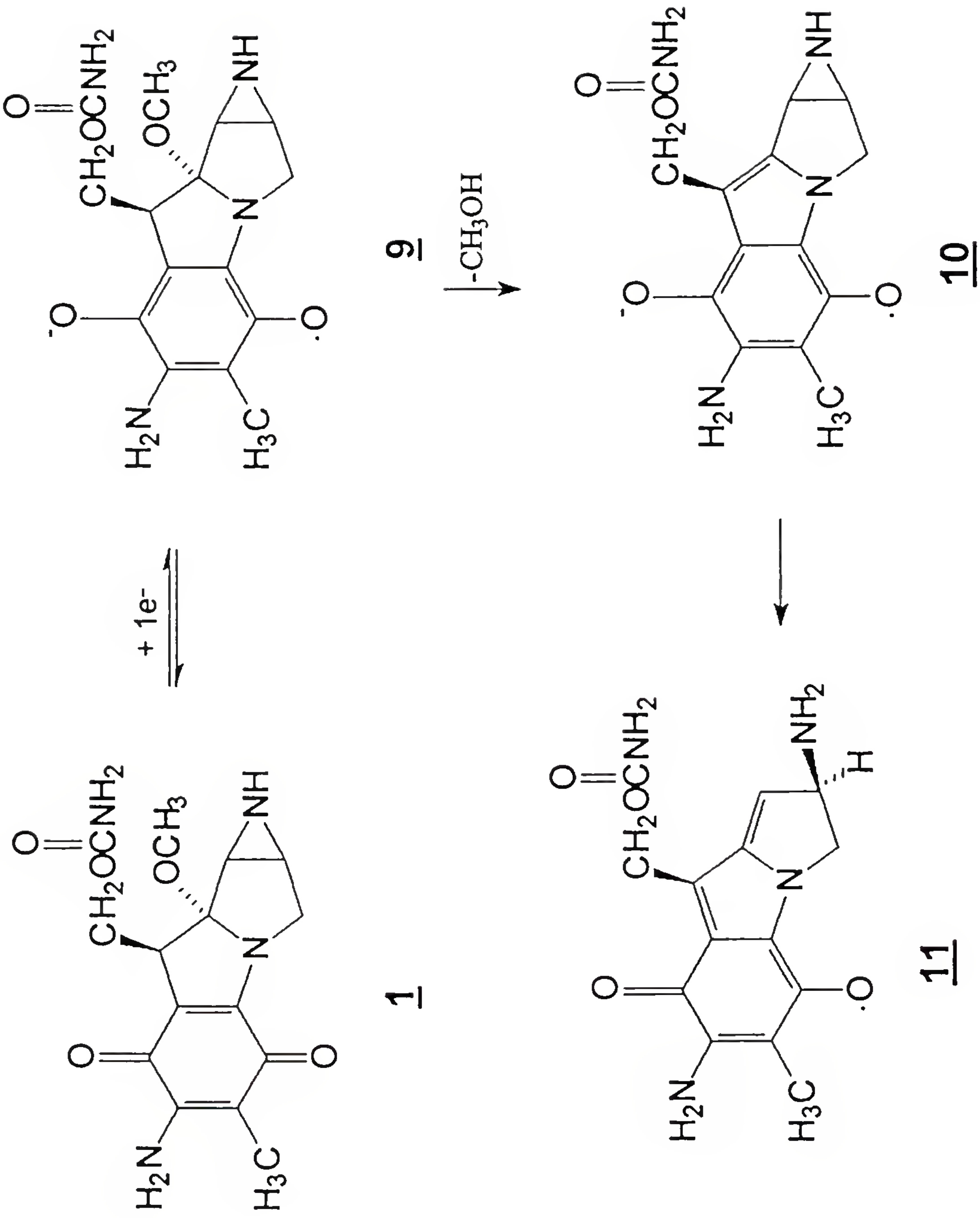

1
2
3
5
$+2e^-, 2H^+$
$-CH_3OH$
H_2O
H_2N
H_3C
OCH_3
CH_2OCNH_2
NH_2
OH
O
N
H
1
2
9
10

Figure 3.4: Proposed mechanism of Mitomycin C reductive activation in Methanol.


+ 1e-
-CH₃OH

MC, that is the loss the of methanol from the reduced intermediate **9** forming **10**, and the three- membered aziridine ring-opening forming **11,** proceeds from the quinone radical stage **9**, which is in agreement with the scheme proposed by Andrews and co-workers for the MC reduction in aprotic DMF.[26]

Graphite Electrodes

In this chapter the electrochemical investigations of MC by cyclic voltammetry (CV) at two different graphite electrode surfaces, glassy carbon (GC) and rough pyrolitic graphite (RPG), will be discussed. The difference in the physical and the chemical properties of GC and RPG accounts for the differences observed in the electrochemical activity of MC at the two surfaces. GC is made by the thermal degradation of organic polymers such as furfural alcohol, phenol formaldehyde and acetone furfural at temperatures of ca. 1800 °C.[62] Pyrolytic graphite (PG) is prepared by the high temperature decomposition of gaseous hydrocarbons such as methane and propane onto a hot graphite surface.[63] The different preparation methods account for the differences in the structures and the properties of the two forms of graphite. Glassy carbon is an amorphous material consisting of interlocking, randomly oriented ribbons[62] (Figure 3.5a). Pyrolytic graphite consists of conjugated hexagonal rings of carbon atoms arranged in planes as schematically illustrated in Figure 3.5b. Before the eletrochemical measurements described here PG is roughened using silicon carbide paper to produce rough pyrolytic graphite (RPG) which produces a surface with high electrochemical activity.

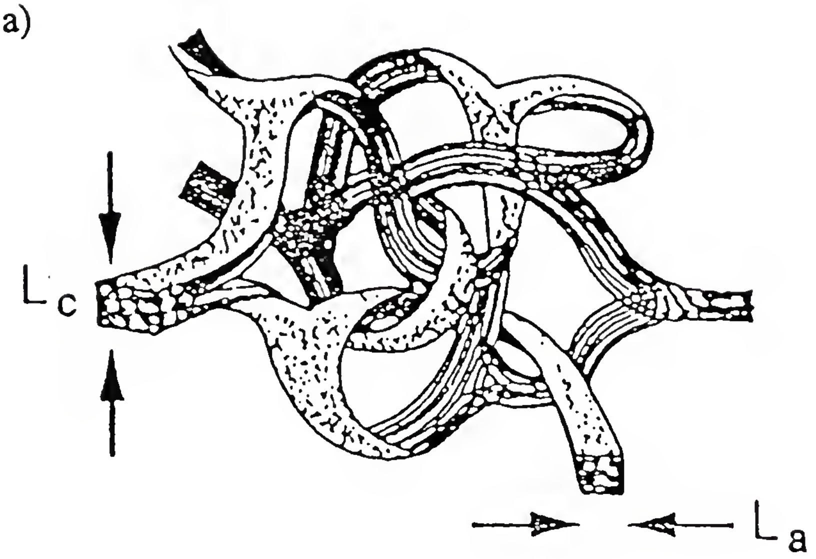
RPG is referred to as an electrochemically active surface while GC is mostly electrochemically inactive unless activated by special treatment procedures. The chemical

Figure 3.5: Structure of Graphite.[62]

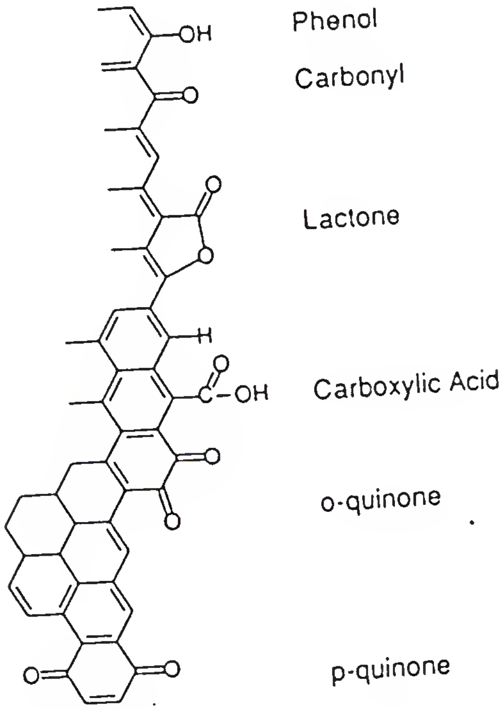
a) Cartoon representation of the Ribbon-Like Structure of Glassy Carbon.

b) Schematic Drawing showing the edge planes with the functional groups and the hexagonal ring structure of the basal plane.

a)


Lc
La

b)


Phenol
Carbonyl
Lactone
Carboxylic Acid
o-quinone
p-quinone

and physical properties related to the chemical activity of these surfaces include [62] : the density of the surface functional groups, surface wettability, double layer capacitance and the surface roughness. The greater activity of RPG over GC is due in part to the high density of theexposed conductive and highly reactive edge planes that are produced during RPG resurfacing compared to GC where the basal plane-like structures dominate.[62,64] This greater activity of RPG is evident from the electrochemical heterogenous rate constants typically obtained for potassium ferricyanide which are one order of magnitude greater at RPG than at GC.[65] Graphite surfaces, because of their high conductivity, low chemical reactivity, low background currents and relatively wide potential windows,[63] are well suited for the electrochemical analysis of biological molecules in solutions which resemble physiological media. Consequently, graphite electrodes were used in the studies which are dicussed later in this chapter.

The electrochemical studies of MC, and the use of the different surfaces in these studies, were first undertaken to identify the optimum parameters for the investigation of MC by electrochemistry on-line with mass spectrometry (EC/MS). The EC/MS technique is uniquely suited to provide insight into biologically relevant, and often very complex, redox activation pathways of drugs such as MC.[42] The interest of this study was to shed light on the activity of one- versus two-electron reduced intermediates towards DNA-adduct formation which could be studied on-line by EC/MS. In general the on-line reductive activation of MC, and the subsequent formation of the adducts of the activated MC with the nucleophiles in solution such as, OH^-, $OCH_3^{-\cdot}$, and guanosine, and their identification by EC/TSP/MS, was expected to be relevant to the understanding of the cancer-fighting ability of MC and related quinone drugs.

The electrochemical cell used on-line in EC/MS had a graphite working electrode.[44] As a result, off-line electrochemistry at both graphite electrodes was first carried out to model the anticipated on-line reactions of MC. An additional important and closely related parameter was the effective operation of the mass spectrometer. For the on-line EC/MS analysis with the thermospray (TSP) interface between the electrochemical cell and the mass spectrometer, the optimization of the electrochemical and the TSP parameters was essential to the acquisition of the mass spectral data with reasonable sensitivities. The ideal conditions for the operation of the TSP interface depend on the compounds to be analyzed. For that reason it was important to have advance information about the expected products from the off-line electrochemical reactions. From the product profiles of MC reduction predicted from off-line electrochemistry experiments it is believed that the optimum on-line conditions for the detection of MC products in EC/TSP/MS can be evaluated. At present the state of the art EC/MS developed by Volk and co-workers using a TSP interface,[42,44] has a time resolution of 50 ms. This provides EC/MS with the unique capability to monitor reactants, short-lived intermediates and products of complex, biologically important reactions,[42] such as relevant in the bioactivity of MC.

Experimental Section

Reagents and Materials

Mitomycin C (MC) was obtained from Aldrich Chemical Co. and as a grant from Bristol Myers Squibb. Solutions of MC were prepared in phosphate buffer and ammonium acetate. MC concentrations in the millimolar range were used to obtain the needed sensitivity in MS detection in the EC/TSP/MS experiments. The typical TSP/MS detection limits are in the micromolar range for MC and its electrochemical reduction products.

Phosphate buffer solutions of 0.05 M, pH 7, were prepared from sodium phosphate monobasic and dibasic (Mallinkrodt). Solution pH was measured using a Corning 130 pH Meter. Relatively low concentrations of phosphate buffer were chosen since these are required for cyclic voltammetric analysis in surfactant solutions which were carried out as part of the electrochemical study of MC. Surfactant assembly at graphite surfaces has been shown to be best at low electrolyte ionic strengths[64]. The surfactant, sodium dodecyl sulfate (SDS), was obtained from Sigma. A 10 mM solution of SDS in 0.05 M phosphate buffer was used. It is important that the SDS concentration be above critical micellar concentration (cmc) of 2.225 mM as in electrochemical experiments at this concentration normal micelles begin to form in solution. Ammonium acetate (0.1 M) was also used in the electrochemical experiments since it was used as the mobile phase in EC/TSP/MS.

Tetrabutylammonium perchlorate (TBAP) was obtained from Aldrich. 0.1 M TBAP was used as the supporting electrolyte in methanol (Fisher) solutions because of its high solubility in methanol compared to the inorganic buffers. All chemicals were used as received and aqueous solutions were prepared in doubly distilled deionized water.

Electrode Preparation and Electrochemical Methods

Rough pyrolytic graphite (RPG) (Electrosynthesis) and glassy carbon (GC) (Electrosynthesis) working electrodes were fabricated by sealing a piece of RPG (ca. 1.5mm diameter X 10 mm length) or a GC rod (ca. 3.0 mm diameter X 5mm length) in glass tubing with inert epoxy (Hysol Aerospace and Instrumental Products Division). The RPG electrodes were resurfaced on 600-grit silicon carbide paper (Mark Laboratories) with a Buchler Ecomet I Polisher-Grinder and rinsed with doubly disstilled deionized water before use. GC electrodes were initially resurfaced with 600-grit silicon carbide paper and were then polished with 0.1 μm gamma alumina particles (Gamal, Fischer Scientific) on a polishing cloth (Mark V Laboratory) using a Buchler Ecomet I Polisher-Grinder. All electrodes were resurfaced prior to each measurement.

The areas of the RPG and GC working electrodes were determined using chroncoulometry with a BAS-100 electrochemical analyzer (Bioanalytical Systems Inc.). A 3.0 mM potassium ferricyanide solution, $K_3Fe(CN)_6$ (Fisher Scientific Co.) in 1.0 mM potassium chloride (Fisher Scientific Co.) was used in these experiments. The areas were calculated from the results using a diffusion coeffient, $D_0 = 7.63 \times 10^6\ cm^2/s$.[66] In the CC experiments the potential step was from 0.400 to 0.0 V versus SCE with a pulse width of 250 ms. The typical electrode areas determined were $0.052 \pm 0.020\ cm^2$ for RPG, and $0.065 \pm 0.020\ cm^2$ for GC. For all electrochemical expweriments data analysis was performed on an IBM PS/2 compatible personal computer. A conventional three electrode set-up was used in the electrochemical experiments. In the all electrochemical experiments RPG or GC was the working electrode with a saturated calomel electrode (SCE) as the reference electrode and a platinum flag auxiliary electrode. All potentials are reported versus SCE at room temperature.

Cyclic voltammetry (CV) experiments were carried out using a BAS-100 electrochemical analyzer with the three electrode set-up. Scan rates, v, in the range of 0.010

to 1.0 V/s were used to measure peak currents (i_p). The difference between the anodic peak potentials (E_{pa}) and the cathodic peak potential (E_{pc}), the peak separation (ΔE_p) was measured at scan rates of 0.010 to 1.0 V. To investigate adsorption at the electrode surface CV experiments were conducted at ν of 0.01 to 0.5 V/s where i_p was measured as a function of ν.

Fundamentals of the Electrochemical Methods

The measured parameters of interest in cylic voltammograms are the anodic peak current i_{pa} and the cathodic peak current i_{pc}, the ratio of anodic to cathodic peak currents, i_{pa}/i_{pc}, and the separation of the cathodic and anodic peak potentials, ΔE_p. For a Nernstain wave with stable product, the ratio of $i_{pa}/i_{pc} = 1$ regardless of scan rate and the diffusion coefficient[67]. Deviation of the ratio of i_{pa}/i_{pc} from unity is indicative of kinetic or other complications in the electrode process such as following chemical reactions. In the case of the following chemical reactions, the product of the electrode reaction reacts with the solvent or other species in solution. The following chemical reactions can affect the forward and reverse electron transfer reaction parameters, therefore affecting the ratio of i_{pa}/i_{pc} and the ΔE_p values. In the presence of a following chemical reaction the ratio $i_{pa}/i_{pc} < 1$ because the electrochemical product is removed from near the electrode surface by the chemical reaction.[67]

The reversibility of an electron transfer process can be determined from the measurement of CV ΔE_p values at different scan rates. The theoretical ΔE_p value for a diffusion controlled heterogenous electron transfer process is 0.059/n V at 25°C,[67] where n is the number of electrons transferred per mole during the process. For an adsorption controlled reversible process the ΔE_p is 0.0V. If the ΔE_p is in the range of 0.061/n V to 0.212/n V for a diffusion controlled process, this is an indication of a quasi-reversible electron transfer kinetics. A ΔE_p value greater than 0.212/n V is indicative of a totally irreversible process.[67]

Electron transfer kinetics can be determined from the ΔE_p values measured at different scan rates. An increase in the ΔE_p values with scan rate is an indication of non-reversible kinetics of the electron transfer process.

In a two-electron, two-proton reduction of an electrochemical species such as the reduction of a quinone to hydroquinone protons will be consumed. The dependence of the electrochemical potential on pH can be shown for a Nernstian system.[68]

$$\mathrm{Ox} + h\mathrm{H}+ + ne^{-} \leftrightarrow \mathrm{Red}$$

$$E = E^{0} - \frac{0.059}{n}\log\frac{[Red]}{[Ox][H^{+}]^{h}} \tag{3.1}$$

By definition pH = -log $[H^+]$ and consequently the electrode potental E is directly related to the pH, giving a change of 0.059 V for every pH unit for a one-electron, one-proton couple. This is a classical behavior for the quinone/hydroquinone system in aqueous solutions.[69]

Measurements of the CV peak current at different CV scan rates can also be used to determine if the process controlling the heterogenous electron transfer involves diffusion or adsorption. The theoretical expression for the CV peak current, i_p, for a reversible diffusion-controlled process is given by the equation:[67]

$$i_p = (2.69 \times 10^5) n^{\frac{3}{2}} A D_o^{\frac{1}{2}} \nu^{\frac{1}{2}} C_o \qquad (3.2)$$

where n, is the number of electrons per mole, A is the electrode area in cm^2 , D_o is the diffusion coefficient in cm^2/s, ν is scan rate in V/s, and C_o^* is the bulk concentration of the electroactive species (mol/cm^3).

For an irreversible diffusion controlled process i_p is given by the equation:[67]

$$i_p = (2.99 \times 10^5) n(\alpha n_\alpha)^{\frac{1}{2}} A C_o D_o^{\frac{1}{2}} \nu^{\frac{1}{2}} \qquad (3.3)$$

where α is the transfer coefficient and n_a is the number of electrons involved in the rate determing step.

In cyclic voltammetry, for an adsorption controlled reversible electron transfer process, i_p is given by the equation:[67]

$$i_p = \frac{n^2F^2}{4RT}\nu A\Gamma_0 \qquad (3.4)$$

where Γ_0 is the surface excess of the electroactive species in mol/cm^2, F is the Faraday's constant (96,484 C), R is ideal gas constant (8.31441 J/K mol) and T is the temperature in K.

For an irreversible adsorption controlled process, i_p is given by the equation:[67]

$$i_p = \frac{n\alpha n_a F^2 A\nu\Gamma_0}{2.718RT} \qquad (3.5)$$

From equations 3.2 and 3.3, for a diffusion-controlled process $i_p \propto \nu^{1/2}$, and from equations 3.4 and 3.5, for an adsorption controlled process, $i_p \propto \nu$. Therefore, a plot of log i_p versus log ν should yield a linear slope with a slope value of 0.5 for a diffusion-controlled process and 1.0 for an adsorption-controlled process. Slope values between 0.5 and 1.0 indicate that both diffusion and adsorption, are involved in the electron transfer process.

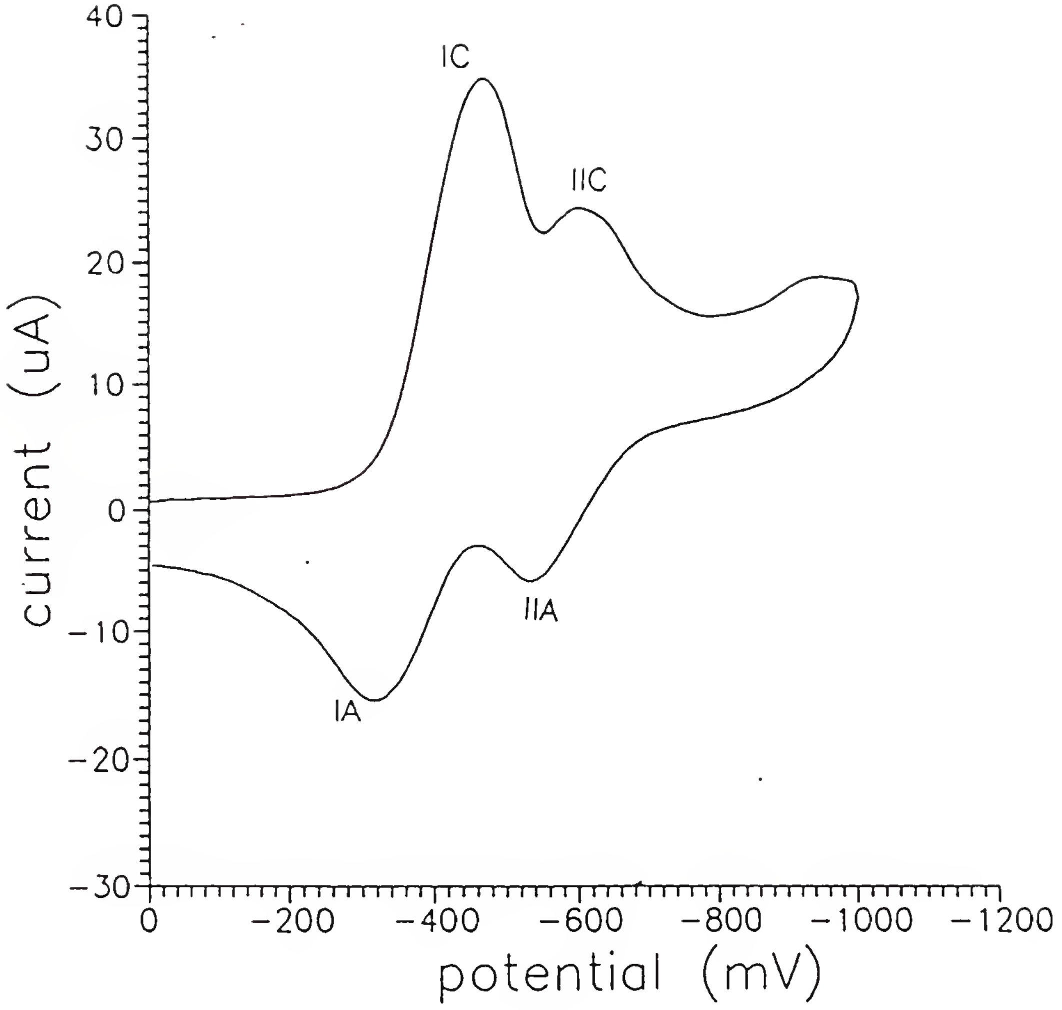
Results and Discussion

Electrochemistry of Mitomycin C in Aqueous Solutions at Graphite Electrodes

Rough Pyrolitic Graphite (RPG) Electrodes

Cyclic voltammetric analysis of MC solutions in pH 7 phosphate buffer at RPG electrodes using moderate scan rates gave two cathodic peaks shown as IC and IIC in Figure 3.6. The peak potentials for each peak are -462 mV and -604 mV, respectively, with peak currents of 37 μA and 22 μA. Two corresponding anodic peaks IIA and IA at potentials of

Figure 3.6: Cyclic Voltammogram of 1.2 mM Mitomycin C in 0.1M Phosphate Buffer pH 7. Scan Rate = 100 mV/s RPG Electrode with Area 0.052 cm^2.


IC
IIC
IIA
IA
current (uA)
40
30
20
10
0
−10
−20
−30
0
−200
−400
−600
−800
−1000
−1200
potential (mV)

-532 and -313 mV with peak currents of 8 μA and 16 μA, respectively, were also observed. The ΔE_p values obtained for the redox couples indicated as I and II, were 149 mV and 72 mV, respectively. The reduction of MC in aqueous solution has been described as a two-electron, two proton diffusion controlled process, [29,30,32] so from the ΔE_p values it can be concluded that process I is totally irreversible while process II is quasi-reversible.

The theoretical peak current for a totally irreversible system is given by equation 3.3. The theroretical peak current for MC in aqueous solution was calculated using the the following values: n=2, $\alpha n_a = 0.5$, A= 0.052 cm^2, $C^* = 1.2 \times 10^{-6}$ mol/cm^3, $D_0 = 5 \times 10^{-6}$ cm^2/s and $\nu = 0.10$ V/s. The calulated peak current (i_p) is 17.94 μA which is below the experimental peak current value of 37 μA obtained for peak IC indicating that possibly adsorption contributes to the experimental current causing current enhancement as a result of surface preconcentration of MC during adsorption.. From the experimental peak current value for peak IIC (22 μA), it can be inferred that the contribution from adsorption is much smalller than for peak IC.

The ratio of the anodic to cathodic peak currents for peaks IC/IA is 0.43 and 0.36 for peaks IIC/IIA. Since these ratios are < 1, this may indicate that chemical reactions are taking place following the electrochemical reduction in addition to the expected effect of the slow kinetics.

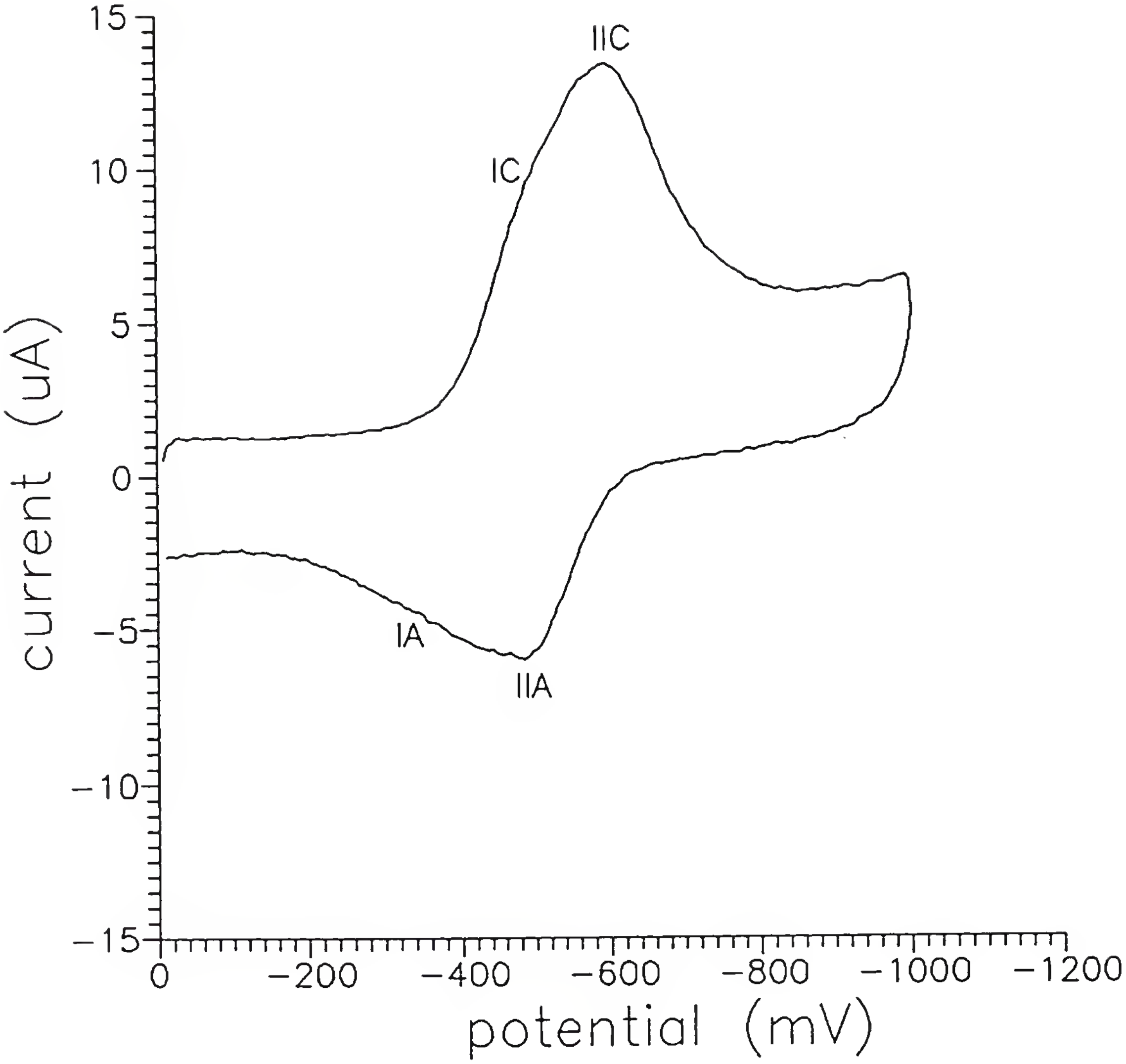
The mobile phase that was used in TSP/MS experiments, ammonium acetate, was also used as an electrolyte in the electrochemical measurements. When 0.1 M ammonium acetate (pH 7) was used as the electrolyte, the cyclic voltammetric analysis of MC yielded similar resluts as in pH 7 phosphate buffer. The two cathodic peaks , IC and IIC, were observed at potentials of -418 mV and -562 mV, respectively. The corresponding two anodic peaks IIA and IA are at potentials of -497 mV and -291 mV. The ΔE_p values were 127 mV and 65 mV for processes I and II respectively. The positive shift in the peak potentials in ammonium acetate electrolyte can be conceivably due to the different surface interactions that can take place in the different electrolytes. Since MC and its electrochemical reaction products can interact with the surface, the electrochemical reaction of MC can be expected to be affected by the type of electrolyte used. The electrolyte had a neglible effect on the peak currents and the separation of peak potentials.

The steeply rising slope of peak IC, observed for MC at RPG (Figure 3.6) compared to that expected for a diffusion-controlled process is indicative of a possible surface mechanism that may involve adsorption. To verify this several experiments were conducted. In the first set of experiments surfactants were used to probe the surface interactions. It has been reported that the addition of an ionic surfactant to a solution containing an adsorbing electroactive species which has some degree of hydrophobicity can decrease the adsorption of that species at graphite electrodes.[70] A surfactant can assemble at the electrode surface forming micellar like aggregates which can compete for the electrode surface with the other adsorbing species in solution and alter the interactions of the adsorbing analyte with the electrode surface. The electrostatic interactions between the surfactant and the electrochemical probe have been shown to be as important to the effective analyte response as the hydrophobic interactions. The electrostatic interactions can presumably allow the probe to access the electrode surface with the surfactant. The probe is no longer directly absorbed at the surface because of the micellar-like assembly which the surfactant can form at the electrode surface. The surface assembly of the surfactant is a dynamic one as are the surfactant-probe interactions.

It was important to verify the nature of the MC interactions with the electrode surface because the electrochemical cell used in on-line EC/MS investigations uses graphite as the working eletrode. If there is adsorption at the graphite surface in the cell used for on-line EC/MS experiments the electrode could eventually become fouled and lose its reactivity which can lead to false interpetation of the MS results.

In order to substantiate the nature of MC interactions with the graphite surface, an anion surfactant sodium deodecyl sulfate (SDS) was chosen. Because SDS is negatively charged and MC is positively charged at pH 7 in phosphate buffer, electrostatic interactions are expected between MC and SDS, while SDS is expected to interact with the electrode surface through a combination of electrostatic and hydrophobic interactions. Cyclic voltammetric analysis of MC in pH 7 phosphate buffer at RPG with 10 mM SDS present in solution yielded a single cathodic peak at -580 mV with a small shoulder at ca. -500 mV and a single anodic peak at -480 mV with a small shoulder at ca. -400 mV (Figure 3.7). In comparing Figures 3.6 and 3.7 it can be seen that following the addition of SDS to the MC solution peaks IC and IA effectively disappear (the processes may be responsible for the small shoulders observed in the presence of surfactant) and the peak potentials of peaks IIC/IIA are shifted ca. 50 mV less negative with the ΔE_p of the IIA/IIC couple increasing by ca. 28 mV to 100 mV. The increase in the ΔE_p can be attributed to the different surface interactions that will occur in the presence of the surfactant.

Figure 3.7: Cyclic Voltammogram of 0.5 mM Mitomycin C in 0.05 M Phosphate Buffer pH 7 with 10 mM SDS added. Scan Rate 100 mV/s. RPG Electrode with area 0.052 cm^2.

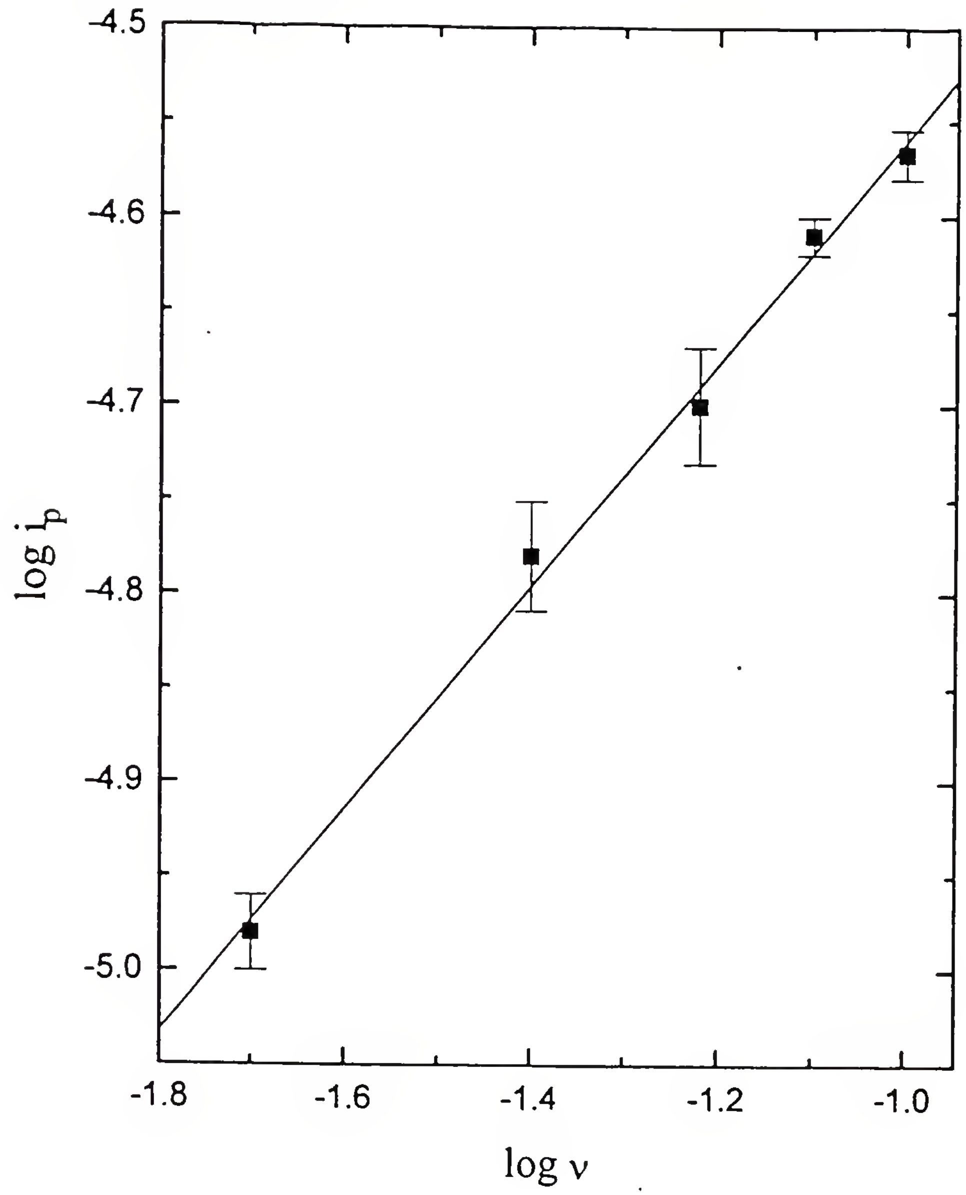

15
10
5
0
−5
−10
−15
current (uA)
0
−200
−400
−600
−800
−1000
−1200
potential (mV)
IIC
IC
IA
IIA

The likely reason for the significant effect of SDS on the response of MC (Figure 3.7) is that SDS can preferably interact with the electrode surface and prevent interactions of MC and its electrochemical products, effectively hindering surface acess by them. This is consistent with the dissappearence of the IC/IA couple and the increase in the ΔE_p for the IIC/IIA couple.

Surface interactions detected from the slopes of log i_p versus log v plots (equations 3.2-3.5) indicate that slope values of ca. 1.0 are due to an adsorption-controlled process, while slopes of ca. 0.5 indicate a diffusion-controlled process. The slopes between 0.5 and 1.0 can signify mixed adsorption-diffusion control. Figure 3.8 shows the log i_p versus log v plot for peak IC in 1.2 mM MC in pH 7 phosphate buffer at the RPG electrode in the absence of SDS. The slope of 0.59 indicates only weak adsorption on the RPG surface.

If the adsorption of the product of an electrochemical reaction is stronger than that of the reactant a pre-peak will be obtained in the cyclic voltammograms of the reactant at more positive potentials than the main diffusion-controlled peak of the reactant [71]. The pre-peak represents the reduction of the dissolved, diffusing reactant to form a layer of adsorbed product, which is an energetically favored process over the reaction of the diffusing reactant[72]. As a result, the reaction forming the absorbing product will occur at lower potentials than the reaction forming a diffusing product. In Figure 3.6 peak IC occurs at a more positive potentials than the process at peak IIC. The two peak CV behavior, together with the results for MC reduction in the presence of SDS where peaks IC/IA effectively disappear, and the calculations of the peak currents which show an enhancement of peak IC current above the theoretical value for a diffusion controlled system, supports contributions

Figure 3.8: Plot of Log Peak Current (i_p) versus Log of Scan Rate (ν) of 1.2 mM Mitomycin C (Peak IC) in 0.05 M Phosphate Buffer pH 7 at RPG Electrode. Slope = 0.590


-4.5
-4.6
-4.7
-4.8
-4.9
-5.0
$\log i_p$
-1.8
-1.6
-1.4
-1.2
-1.0
$\log \nu$

of adsorption at peaks IC/IA while peaks IIC/IIA are due to a diffusion-controlled process.

Glassy Carbon (GC) Electrodes

Glassy carbon (GC) electrodes have a smoother and less active surface than the RPG electrodes[62]. GC electrodes were also used for the cyclic voltammetric analysis of MC. Since the electrochemical cell used on-line in the EC/MS experiments the working electrode was reticulated vitreous carbon which has properties similar to those of GC[62]. Results of the cyclic voltammetric analysis of MC in pH 7 phosphate buffer at GC electrodes at scan rates of 100 mV/s showed the presence of one electroreduction process (Figure 3.9). The potential of the cathodic peak labelled here as IC was -540 mV, and the anodic peak, labelled here as IA was at a potential of -100 mV. This compares to potentials of -462 mV and -604 mV for the cathodic peaks and potentials of -532 mV and -313 mV for the anodic peaks at RPG electrodes. The ΔE_P value of 440 mV at GC indicates a totally irreversible electron-transfer process. A plot of log i_p versus log ν provided a slope of 0.49 indicating diffusion-control of the electron transfer process at GC (Figure 3.10). The CV peak currents (i_p) observed at GC electrodes are 38 μA for the cathodic i_p and - 8.5 μA for the anodic i_p. The theoretical value of the peak current the GC electrode (area 0.0752 cm^2) was calculated as 27 μA using equation 3.2. Thr current enhancement observed in this case is not due to adsorption. The ratio of i_{pa}/i_{pc} of 0.22 suggests that irreversible chemical reactions take place following the initial electrochemical reduction.

The electrochemical behavior observed at RPG and GC electrodes is consitent with the two-electron, two-proton reduction of the quinone moiety to the hydroquinone (Figure 3.1) as described for aqueous solutions by Rao and co-workers at mercury electrodes[32]. At

Figure 3.9: Cyclic Voltammogram of 1.2 mM Mitomycin C in 0.05 M pH 7 Phosphate Buffer at GC Electrode. Scan Rate 100 mV/s. Electrode Area 0.075 cm^2.

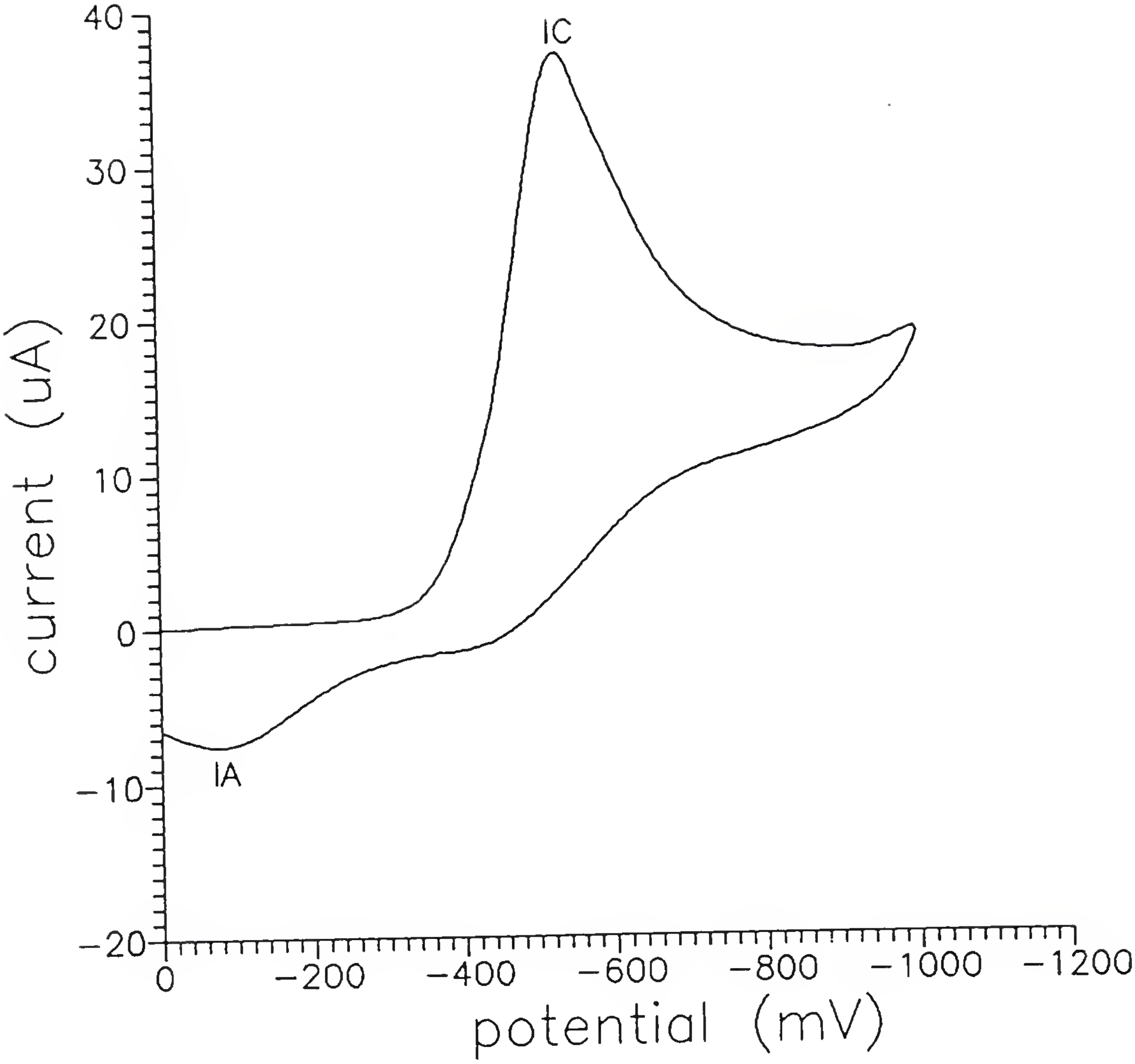
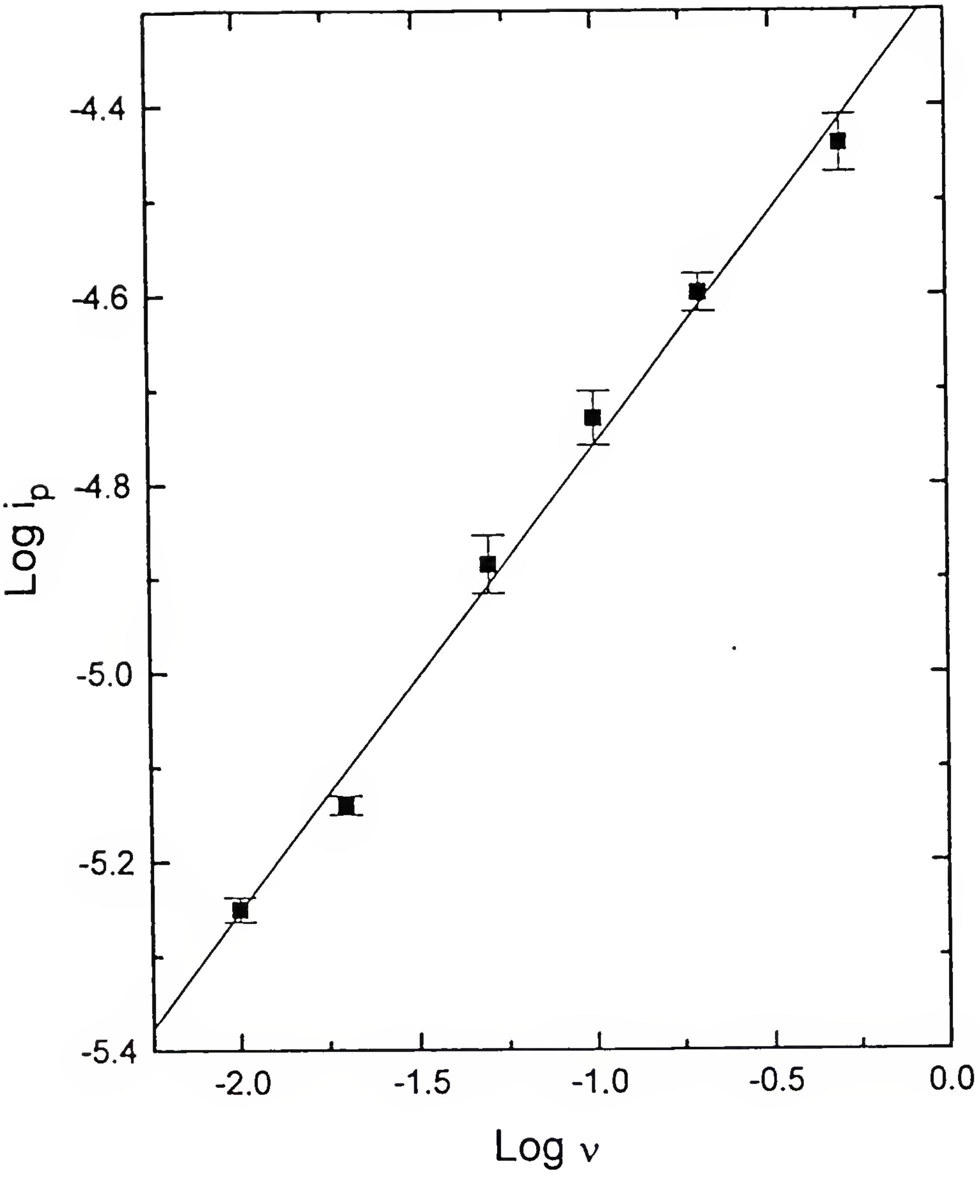

IC
IA
current (uA)
40
30
20
10
0
−10
−20
potential (mV)
0
−200
−400
−600
−800
−1000
−1200

Figure 3.10: Plot of Log Peak Current versus Log Scan Rate of 1.2 mM Mitomycin C in 0.05 M Phosphate Buffer pH 7 at GC Electrode. Slope = 0.495.


-4.4
-4.6
-4.8
-5.0
-5.2
-5.4
Log i_p
-2.0
-1.5
-1.0
-0.5
0.0
Log ν

RPG, peaks IC and IA are likely due to the adsorption of the product formed in the reduction of MC while peaks IIC and IIA are likely due to the diffusion controlled reduction of the quinone since these peaks are still observed in the presence of SDS. At GC no adsorption is indicated from the scan rate studies, and peak IC can be assigned as due to the diffusion-controlled reduction of the quinone to the hydroquinone. The reduction at GC is slower than at RPG and will be more affected by the following chemical reactions which can occur in aqueous solutions and which can account, in addition to reaction irreversibilty, for the small ratio of i_{pa}/i_{pc} determined for MC reduction at GC and for observed enhancement of peak IC currents.

Electrochemistry of Mitomycin C in Methanol at Graphite Electrodes

Cyclic voltammetric analysis of 3.5 mM MC in methanol with 0.1 M TBAP electrolyte at both RPG and GC electrodes shows the presence of three cathodic peaks IC, IIC and IIIC and two anodic peaks IIA and IIIA, at scan rates of 100 mV/s (Figures 3.11 and 3.12). The significant differences in response observed for MC in aqueous solutions at RPG compared to GC are not seen in methanol. However, in the two solvents the responses of MC are significantly different, as seen by comparing Figures 3.6 and 3.11. Since in methanol, the response of MC at RPG and GC is similar, and all processes observed at RPG are observed at GC only the experiments on RPG will be discussed. Since similar results are obtained at GC but the peaks at RPG are better defined than at GC presumably because, even in methanol, RPG is a more active surface.

Peak IC at a peak potential of -600 mV with a peak current of 65 μA has no reverse counterpart at 100 mV/s while peaks IIC (-685 mV, 64 μA) and IIIC (-820 mV, 66 μA) show

Figure 3.11: Cyclic Voltammogram of 1.2 mM Mitomycin C in Methanol with 0.1 M TBAP Supporting Electrolyte at RPG Electrode. Scan Rate 100 mV/s. Electrode Area 0.055 cm^2.

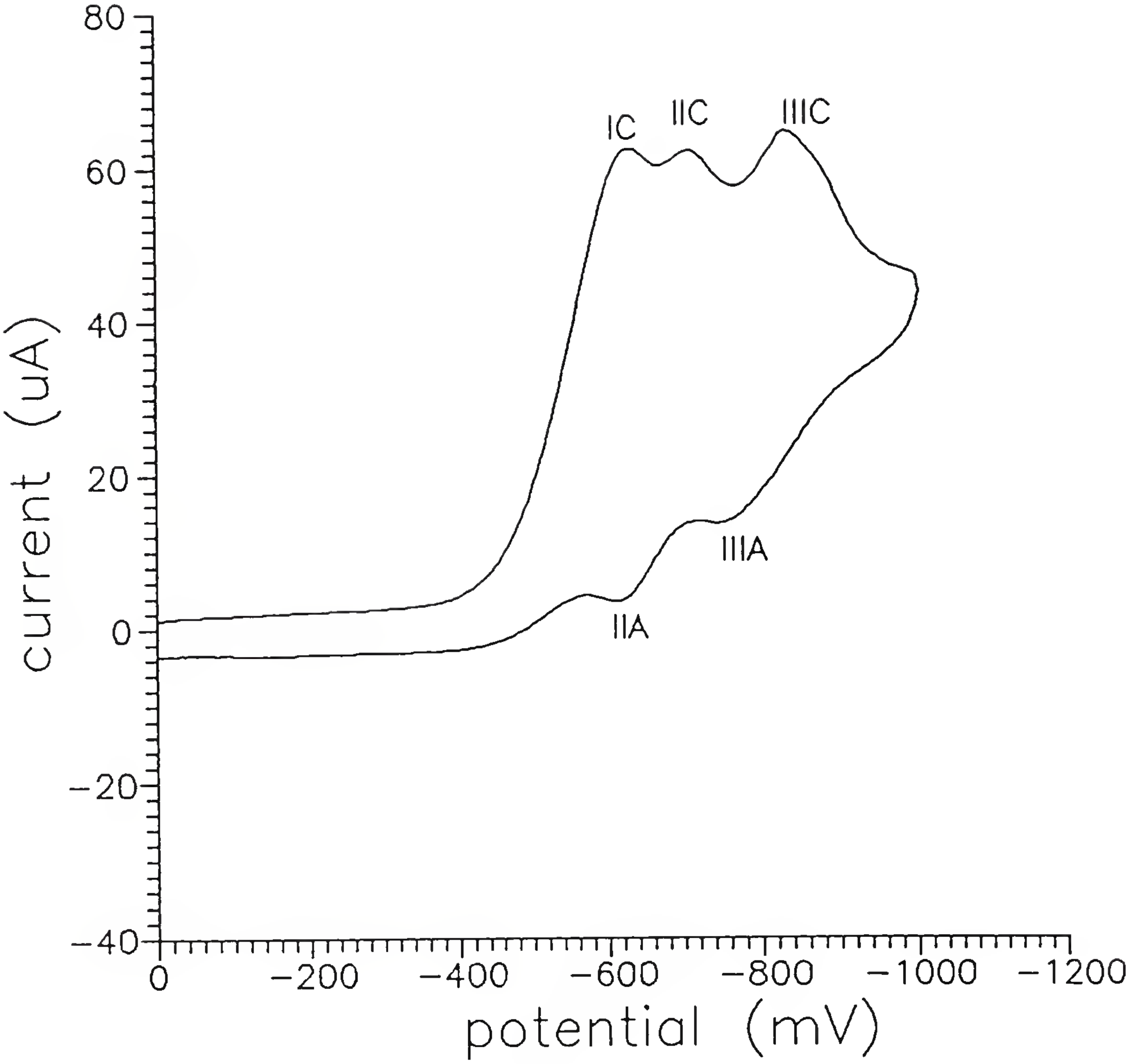
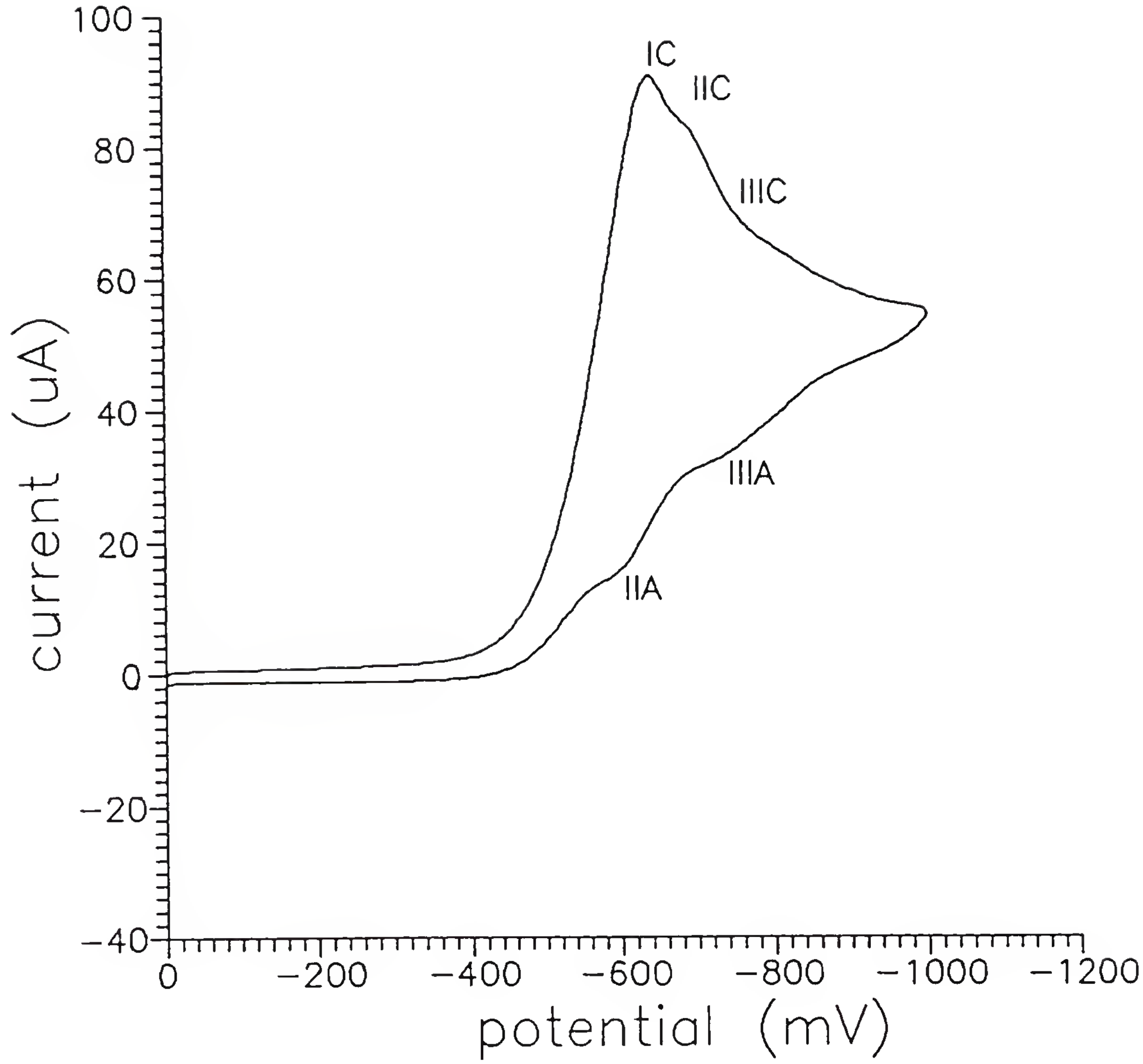

current (uA)
80
60
40
20
0
−20
−40
potential (mV)
0
−200
−400
−600
−800
−1000
−1200
IC
IIC
IIIC
IIA
IIIA

Figure 3.12: Cyclic Voltammogram of 1.2 mM Mitomycin C in Methanol with 0.1 M TBAP Supporting Electrolyte at GC Electrode. Scan Rate 100 mV/s . Electrode Area 0.075 cm^2.

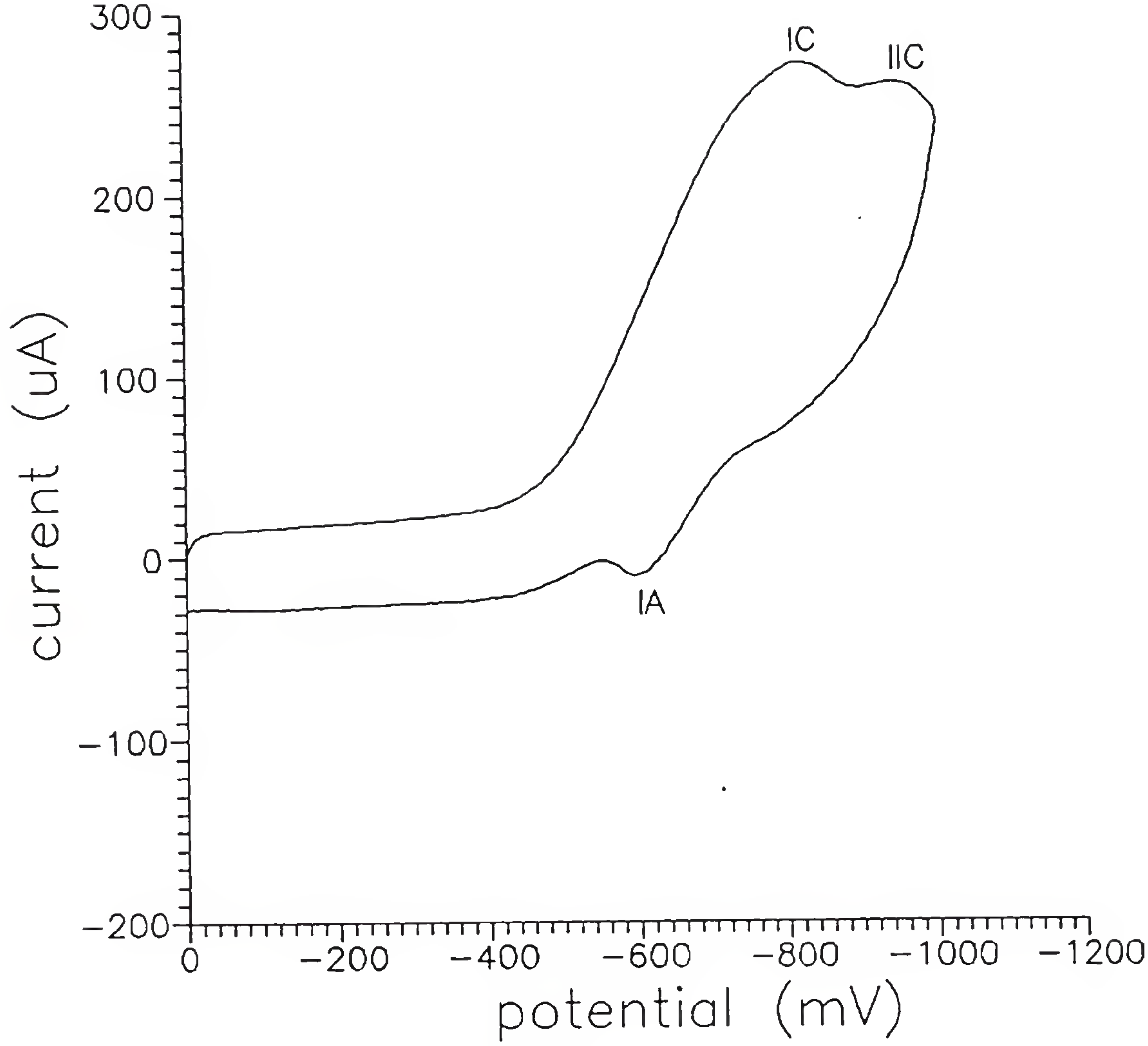

current (uA)
100
80
60
40
20
0
−20
−40
0
−200
−400
−600
−800
−1000
−1200
potential (mV)
IC
IIC
IIIC
IIIA
IIA

reverse peaks. For the two processes (II and III) which show the reverse peaks the ΔE_P values are 75 and 80 mV, indicating quasi-reversible diffusion-controlled behavior. The theoretical diffusion peak current calculated using equation 3.2 for peak IC, assuming a two-electron process with one-electron involved in the rate determining step, is 52.12 μA which is comparable to the values obtained experimentally.

The electrochemical reduction of quinones in non-aqueous media is known to involve the initial formation of an anion radical prior to the formation of the semiquinone and the hydroquinone species.[73] In aqueous solutions the overall reduction of MC is a two- electron, two-proton process. The effect of hydrogen ion concentration on the shift in the reductive peak potentials in aqueous solutions has been described by Rao and co-workers.[32] They reported that the pH dependence of the potentials corresponds to the theoretical slope of 61.7 mV/pH unit expected for a two-electron, two-proton process. The shifts confirmed that protons were involved in the reduction of MC in aqueous solutions.[32] Kohn and coworkers suggested that since the reduction potential of MC in methanol is independent of pH,[28] supported the conclusion that the reduction led to the formation of the quinone radical anion since the H^+ ions were not apparently involved in the reaction. From the cyclic voltammetric and the ESR spectroscopy analysis it was proposed that the opening of the three-membered arizidine ring proceeded from the quinone radical stage as opposed to the hydroquinone stage as proposed for non-aqueous media by Andrews and coworkers.[26]

Cyclic voltammetric analysis of MC in methanol at scan rates of 1.0 V/s yielded two cathodic peaks IC and IIC, and one anodic peak IA (Figure 3.13). It can be concluded that

Figure 3.13: Cyclic Voltammogram of 1.2 mM Mitomycin C in Methanol with 0.1 M TBAP Supporting Electrolyte at RPG Electrode. Scan Rate 1000 mV/s Electrode Area 0.055 cm^2.


current (uA)
300
200
100
0
−100
−200
potential (mV)
0
−200
−400
−600
−800
−1000
−1200
IC
IIC
IA

the anodic peak IA is the reverse process to the reduction peak IC and not to the peak IIC. This is because at faster scan rates of 1V/s when peak IIC decreases, the reverse peak labelled here IA, increased as is expected for the reverse process for which the forward peak also increases. Peak IIC is due to the same process as the peak IIC observed at 100 mV/s. Comparison of the results at 1 V/s to those obtained at 100 mV/s supports the formation of the radical anion in process I. At 1V/s the redox reaction of the radical anion can be expected to be more reversible, and as expected, the radical oxidation is supported by the presence of peak IA. At 100 mV/s, the radical reduction process IC is irreversible, because the chemical reactions, including nucleophilic addition that can occur following the electron transfer, have more time to remove the radical from solution. This is further supported by the decrease in peak current of peak IIC at 1 V/s compared to the current measured at 100 mV/s. According to equations 3.2-3.5 the peak current increases with the scan rate. However this will not be observed if the peak current is due to the product formed in a relatively slow chemical reaction from a product of the electrochemical electron transfer reaction.

A pathway for the reduction of MC in methanol proposed by Kohn and coworkers,[28] for the reduction at platinum and mercury electrodes (Figure 3.4) can be used to explain the cyclic voltammetry results obtained here in methanol (Figure 3.11). The redox process labelled IC in Figure 3.11 can be attributed to the formation of the radical anion **9**. The loss of the methoxy group follows forming **10**. The subsequent opening of the strained three-membered aziridine ring can form the biologically active form of MC **11**, where the addition of the nucleophile OCH_3^- in trans and cis configurations can form **15**. **15** can be reoxidized to **16** at the electrode surface or by oxygen present in solution. The addition of the nucleophile OCH_3 is presumably the irreversible chemical reaction that is observed in the CV experiments following the reduction of MC (**1** - **9**, Figure 3.4) at peak IC (Figure 3.11). It has been suggested by Kohn and co-workers that the peaks, assigned here IIC/IIA and IIIC/IIIA, can be conceivably due to the step-wise formation of the semiquinone and the hydroquinone species of **16**. In the CV experiments **16** is reduced to **15** as the potential becomes more negative, leading to the processes IIC/IIA and IIIC/IIIA which are observed by cyclic voltammertry.

Conclusions

Based on the previous product studies of MC following reductive activation, the reduction mechanism of MC at pH 7 is considered an electrochemical-chemical-electrochemical reaction, where the chemical reaction represents one or more steps. This was confirmed from the electrochemical studies in water and methanol at graphite electrodes. The electrochemical steps are considered reversible while in methanol the chemical steps are considered irreversible in both solvents. The electrochemical activation of MC is followed by a series of irreversible chemical reactions including the addition of a nucleophile. The observed differences in peak potentials are partly affected by the chemical reactions. When the intervening chemical reactions are slow there is little or no effect on peak potentials but if the chemical reactions are fast, there will be a shift in the reduction peak to more positive potentials[71,72]. This phenomenon is due to the removal of the electrochemical product by the chemical reaction. This increases the electrochemical reaction rate and leads to a larger reduction current at more positive potentials.

The electrochemical studies of MC in methanol suggest that the initial one-electron reduction step, leads to the subsequent loss of methanol and the opening of the three-membered arizidine ring and therefore, the chemical changes proceed from the quinone radical stage. These results are corroborated by the work of Andrews and co-workers[26], Danishefsky and Egberton [74] and Kohn and coworkers.[28]

The type of solvent used in the MC reduction is responsible for the products formed. In aqueous solution, the hydroxide ion acts as a nucleophile and is added to MC, while in methanol the methoxy ion is added. These ions are involved in the competitive reactions with other possible nucleophiles in solution and therefore can form a mixture of products. The main products of the nucleophilic reactions in aqueous solution are expected to be cis and trans hydroxide substituted MC derivatives which have lost a methoxy group and in which the strained three-membered arizidine ring is opened, while in methanol this will be the methoxy substituted product.

CHAPTER 4
THE REDUCTIVE ACTIVATION OF MITOMYCIN C BY ELECTROCHEMISTRY ON-LINE WITH MASS SPECTROMETRY

Electrochemistry On-line with Mass Spectrometry

The on-line combination of electrochemistry and mass spectrometry (EC/MS) can provide information about electrochemically generated species with excellent time resolution. This technique can be used to provide insights into biological redox reactions. Electrochemistry can be used to model complex biologically important enzymatic redox reactions, while the mass spectrometer is used as a tool for chemical identification of reactants, intermediates and products of these reactions. Other methods such as spectroelectrochemistry, where electrochemical reactions are monitored by UV-vis spectroscopy, have been used to obtain chemical information about electrochemical reactions.[75]

The inherent sensitivity and selectivity of mass spectrometry allows for the identification of individual components in even very complex biological matrices which may be difficult to accomplish by spectroscopic methods because of interferences. The use of GC/MS to obtain chemical information about electrochemical reactions can be tedious.[76] Dryhurst and coworkers combined several techniques to investigate the oxidation pathway of uric acid with final final identification by GC/MS.[34] Electrochemical measurements were combined with UV-vis spectroelectrochemistry, gel-permeation chromatography, liquid chromatography, and off-line GC/MS. Prior to analysis by GC/MS the electrochemically generated species had to be derivatized.

The development of the thermospray interface (TSP) by Vestal and Blakely [39] has allowed for the on-line combination of EC/MS. Hambizter and Heitbaum were the first to use the thermospray interface to couple an electrochemical cell to a mass spectrometer.[43] In these experiments the on-line electrochemical oxidation of N,N-dimethylanaline (DMA) was monitored and the electrochemical products of the oxidation reaction were detected by mass spectrometry. The time resolution between the electrochemical generation and detection by the mass spectrometer was ca. 10 seconds. The state-of-the-art EC/TSP/MS developed by Volk and coworkers has a time resolution of ca. 500 ms,[42,44] which allows for the detection of short lived intermediates formed in the electrochemical reactions. The intermediates and products resulting from the electrochemical oxidation of uric acid were monitored and characterized by this method. Tandem mass spectrometry was important in determining the structures of the electrochemically generated species.[77] With tandem mass spectrometry, Volk and coworkers were able to obtain structural information about each component in the electrolysis mixture without chromatographic separation.[42,44]

Getek and coworkers used EC/MS with a thermospray interface to monitor the reaction of acetaminophen with glutathione after the electrochemical oxidation of acetaminophen.[78] The formation of conjugates of acetaminophen and glutathione occurs *in vivo*, after oxidation of acetaminophen to a reactive species. The mixing of glutathione with the oxidized acetaminophen resulted within one second in the formation of the conjugate.

The focus of this chapter is on the EC/TSP/MS investigation of the electrochemical reduction of mitomycin C (MC) specifically the formation of adducts with the DNA base guanosine following the electrochemical reductive activation of MC. On-line electrochemistry was used to reductively activate MC and the mass spectrometer was used to monitor the reactions of the reductively activated MC, including the formation of an adduct with guanosine.

Experimental

Samples and Reagents

Mitomycin C was obtained from Sigma, and as a grant from Bristol Myers Squibb. Analytical grade ammonium acetate was obtained from Fisher Scientific. HPLC grade methanol was obtained from Fisher. Mitomycin C was dissolved in, and injected into either 0.1 M ammonium acetate or 0.1 M ammonium acetate/methanol (50/50 v/v). Solutions of MC in the 10-500 mM concentration range were prepared in these solvents. Guanosine and deoxyguanosine were obtained from Sigma. Solutions of guanosine and deoxyguanosine were prepared in 0.1 M ammonium acetate at concentrations of 250 mM.

Mass Spectrometry

A Vestec Model 1201 dedicated LC-MS (Vestec Corp. Houston TX) with a thermospray interface was used in all thermospray mass spectrometry. The mobile phase was delivered by an Altex model 110a HPLC pump at flow rates ranging from 1.0 to 2.5 mL/min. Samples were injected with a Rheodyne (model 7410) injector fitted with a 100 μL injection loop into the flowing mobile phase. All solutions were filtered and deareated prior to use. The mobile phase was either the 0.1 M ammonium acetate or the 0.1 M ammonium acetate/methanol (50/50 v/v) solution.

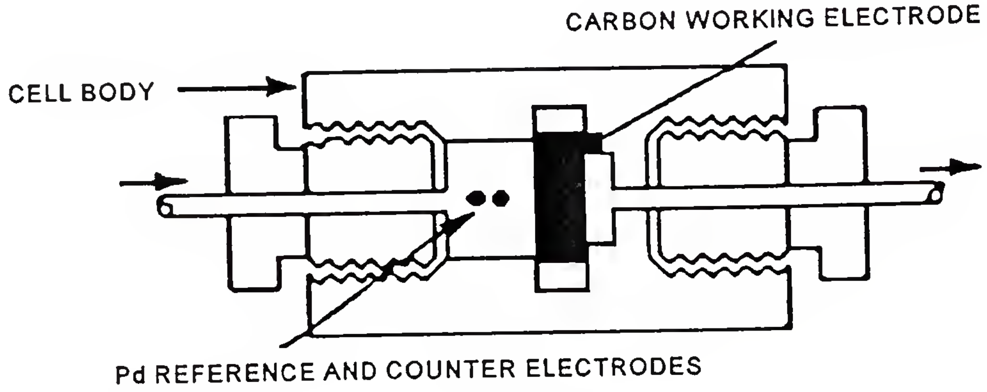
The vaporizer exit temperature (tip temperature) is critical in the acquisition of thermospray mass spectral data. The tip temperature along with the source block temperature were monitored in the experiments. At typical flow rates of 1.5 mL/min in 0.1 M ammonium acetate the operating temperatures were: tip, 240 °C and source 298 °C. When the mobile phase was changed to the 0.1 M ammonium acetate/methanol (50/50 v/v) solution the optimum operating temperates were: tip, 210° C, source 298° C. A 500 ppm of polyethylene glycol (PEG 200) in 0.1 M ammonium acetate was used to calibrate the thermospray mass spectrometer. In all thermospray mass spectrometric experiments were carried out as flow injection experiments where the analytes were injected into the flowing mobile phase.

Electrospray mass spectrometry was carried out on a Vestec 201 mass spectrometer with an electrospray interface. Mobile phase of methanol/water (50/50 v/v) solution acidified with HCl, at flow rates of 5.0 μL/min was used in all electrospray mass spectrometric studies.

On-Line Electrochemistry

The on-line electrochemical results were obtained using an ESA model 5020 coulometric electrochemical cell (ESA, Inc. Bedford MA) (Figure 4.1). The electrochemical cell (EC cell) has a cell volume of ca. 50 μL with a reticulated vitreous carbon working electrode (reticulated vitreous carbon is similar in performance to glassy carbon) with an area of ca. 12 cm^2. In this cell the auxiliary and reference electrodes are palladium wire. The palladium reference electrode is a quasi-reference with potentials within ca. 50 mV of SCE. The electrochemical cell is rated to function at pressures of up to 6000 psi. A model 173

Figure 4.1: ESA Electrochemical Cell for On-Line EC/MS


CARBON WORKING ELECTRODE
CELL BODY
Pd REFERENCE AND COUNTER ELECTRODES

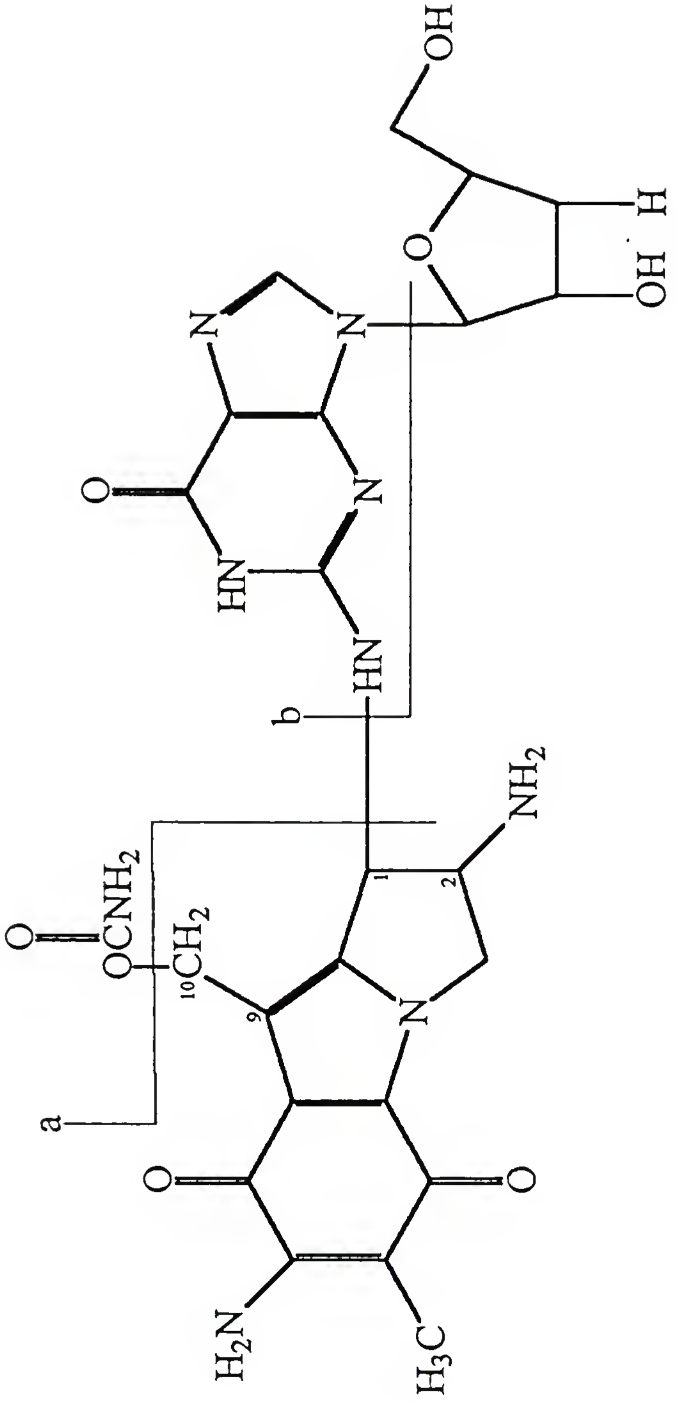
PAR potetentiostat (EG&G Princeton Applied Research, Princeton, NJ) was used to control the on-line electrochemical cell. The large ratio of the working electrode area to the cell volume allows for significant electrochemical conversion efficiencies of ca. 85% as reported by Volk and co-workers for uric acid in EC/TSP/MS experiments.[42,44,77]

Thermospray Mass Spectrometry of Mitomycin C

The mass spectral analysis of MC by electron impact, chemical ionization, field desorption and fast atom bombardment mass spectrometry has been reported.[24,79] Musser and Callery reported the LC thermospray mass spectral analysis of the MC-deoxyguanosine adduct.[31] The adducts which were formed from the reaction of MC with calf thymus DNA were separated from the unmodified nucleosides by HPLC and microgram quantities of the adducts were identified by thermospray mass spectrometry.

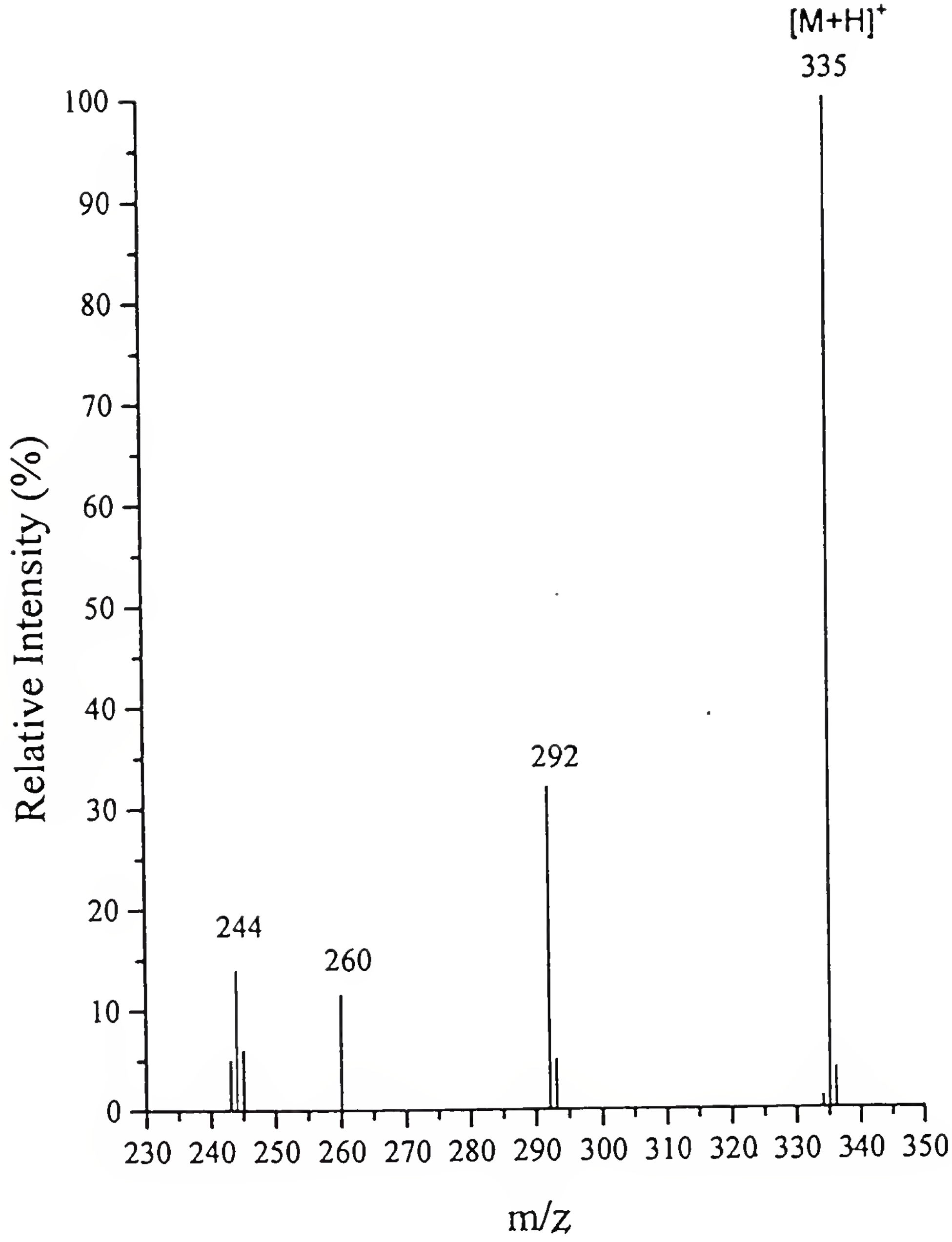
In order to investigate MC with EC/TSP/MS experiments were first carried out to determine the thermospray mass spectral characteristics of MC such as fragmentation patterns. When a solution of 30 mM MC in 0.1 M ammonium acetate was used in thermospray MS analysis in the initial attempts in this work there was considerable fragmentation and the molecular ion of MC had very low abundance or was not detected. The base peak of m/z 244 which has been previously observed in the thermospray mass spectrometry of MC- deoxyguanosine adducts,[31] was obtained for MC in 0.1M ammonium acetate is characteristic of both MC and its adducts (Figure 4.2). Thermospray mass spectral analysis of MC-deoxyguanosine adduct by Musser and Callery produced a fragment ion at m/z 244.[31] In order to follow the reduction of MC by EC/TSP/MS the molecular ion or an ion characteristic of MC only must be monitored. Figure 4.3 shows the thermospray mass

Figure 4.2 : Thermospray MS Fragmentation of Mitomycin C-Deoxyguanosine Adduct:[31]
a) Prontonated MC segment m/z 244
b) Guanine Base $[B+2H]^+$ m/z 152.



OH
O
OH
H
N
N
O
N
HN
HN
b
NH$_2$
1
2
O
OCNH$_2$
^{10}CH$_2$
9
a
N
O
O
H$_2$N
H$_3$C

Figure 4.3 Thermospray Mass Spectrum of Mitomycin C: Mobile phase 0.1 M Ammonium Acetate/Methanol 50/50 v/v. Flow rate 1.5 mL/min. Tip temperature 210 °C.


[M+H]$^+$
335
292
244
260
Relative Intensity (%)
0
10
20
30
40
50
60
70
80
90
100
230
240
250
260
270
280
290
300
310
320
330
340
350
m/z

spectrum of 150 mM MC in 0.1 M ammonium acetate/methanol (50/50 v/v). The base peak at m/z 335 is due to the protonated molecular ion $[M+H]^+$. The assignment of fragments 292, 260 and 244 is shown in Table 4.1.

An apparent correlation between the extent of MC fragmentation and the composition of the mobile phase was observed when mass spectral data for MC were obtained. Figure 4.4 shows the relationship between the composition of the mobile phase and the abundance of the pseudo-molecular ion at m/z 335. As the amount of methanol increases in the mobile phase the abundance of the MC pseudo-molecular ion m/z 335 increases. Eventually the pseudo-molecular ion abundance decreases after the amount of methanol in the mobile phase reaches 50%. The difference in specific heat of water and methanol is likely responsible for the observed changes in the molecular ion abundance because methanol which has a lower specific heat can vaporize at a lower temperature. At lower vaporization temperatures MC can undergo less fragmentation. The decrease in m/z 335 when the methanol concentration is greater than 50 % occur because the discharge electrode of the thermospray mass spectrometer used to aid ionization operates adequately only in primarily aqueous mobile phases.

The optimum tip temperature for the acquisition of the mass spectra of MC was obtained by maximizing the MC intensities for m/z 335 at a given flow rate. The increase in the amount of methanol in the mobile phase leads to changes in the optimum tip temperature for the acquisition of mass spectral data of MC. Figure 4.5 shows the variation of the optimum tip temperature with a change in the mobile phase composition at a flow rate of 1.5 mL/min. The base peak ions obtained in the thermospray mass spectral analysis of MC were

Table 4.1 Summary of Mass Spectral Data for Mitomycin C

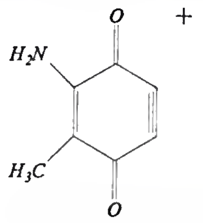
	m/z	Assignment
TSP (Figure 4.3)	335	$[M+H]^+$
	292	$[M\text{-}OCNH_2]^+$
	260	$[(M\text{-}O_2CNH_3)\text{- }CH_3]^+$
	244	$[(M\text{-}CH_3O)\text{-}O_2CNH]^+$
ESP (Figure 4.10)	691	$[2M+Na]^+$
	335	$[M+H]^+$
	274	$[M\text{-}O_2CNH_2]^+$
	242	$[(M\text{-}CH_3\,OH)\text{-}O_2CNH_2]^+$
	135	

Figure 4.4 Response of Mitomycin C vs Methanol Content of the 0.1 M Ammonium Acetate Mobile Phase. Flow Rate 1.5 mL/min. Tip Temerature 220 °C.

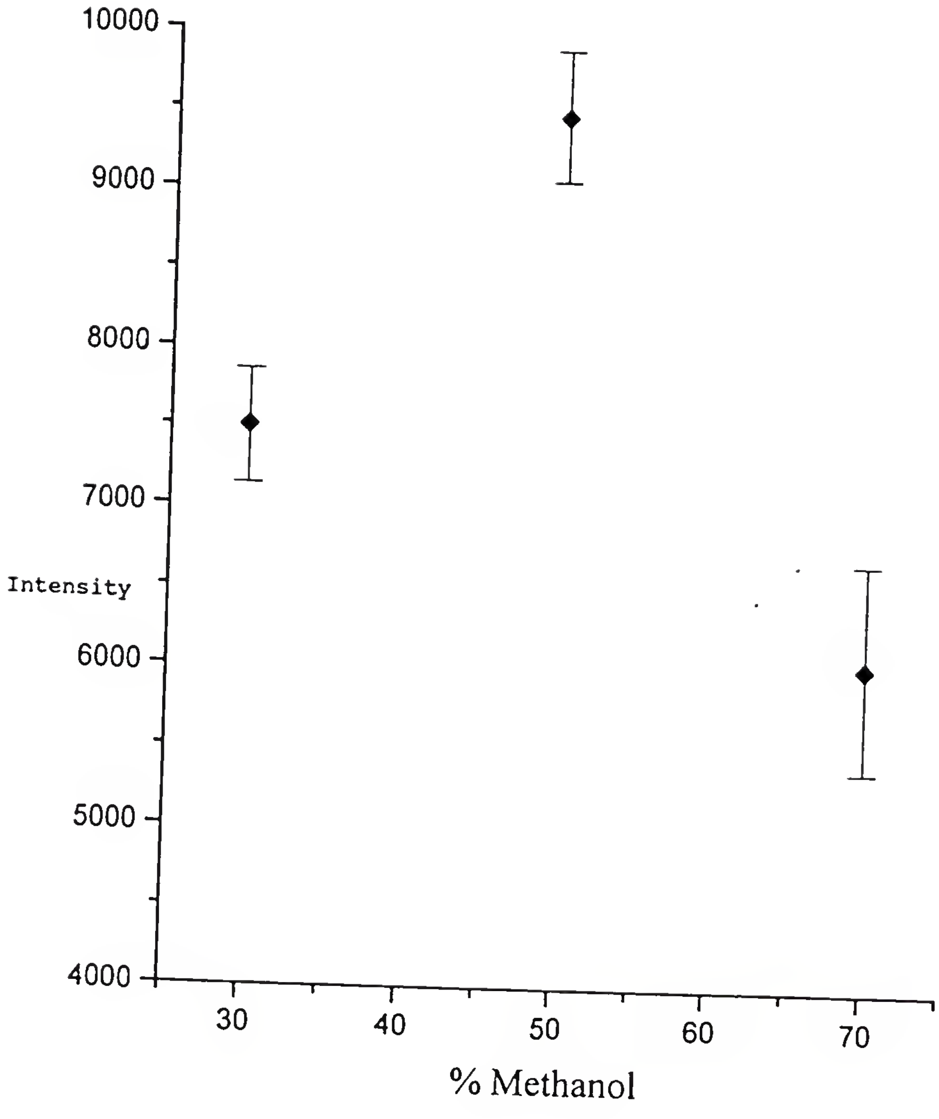
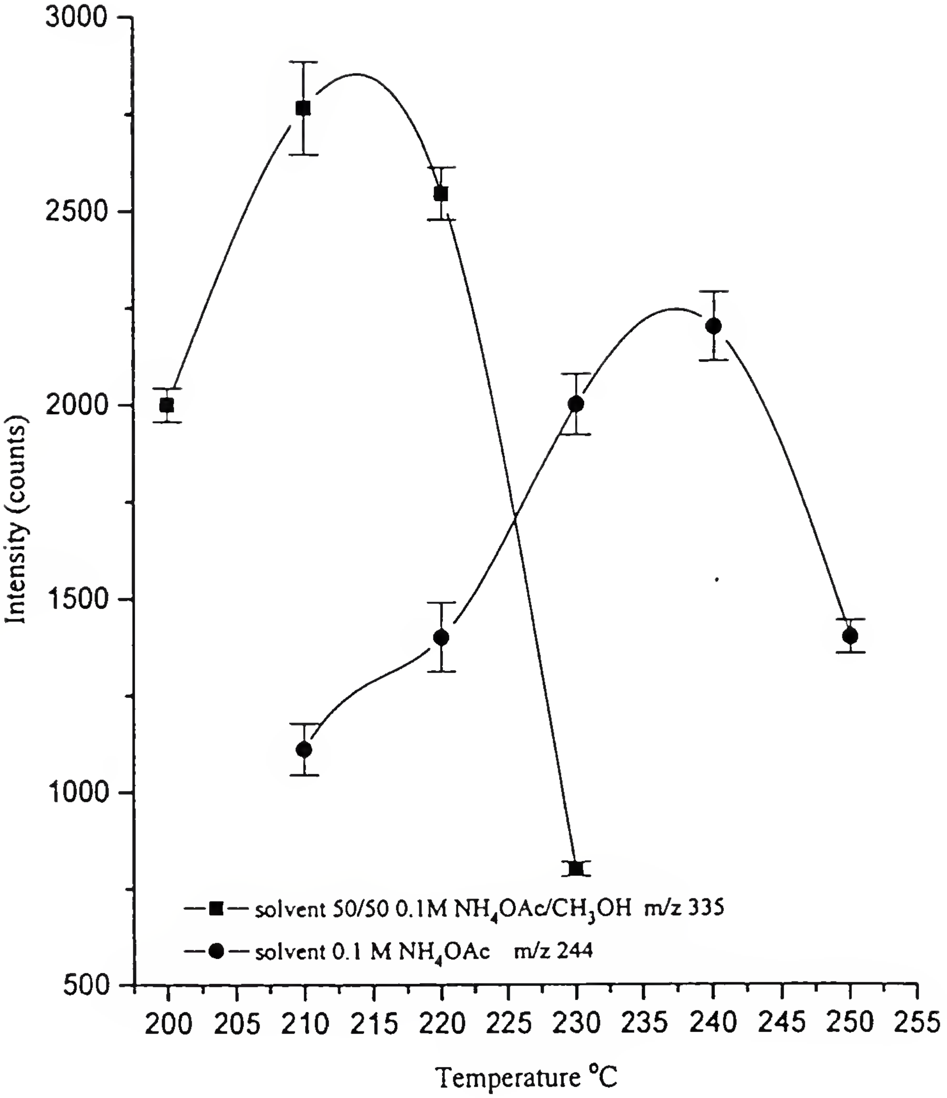

10000
9000
8000
7000
6000
5000
4000
Intensity
30
40
50
60
70
% Methanol

Figure 4.5: Response of Mitomycin C Thermospray Base Peak Ion vs Tip Temperature. Flow Rate 1.5 mL/min Tip Temperature 210 °C for 50/50 Methanol/ 0.1 M Ammonium Acetate Solvent. Tip Temperature 240 °C for 0.1 M Ammonium Acetate Solution.



Intensity (counts)
3000
2500
2000
1500
1000
500
200
205
210
215
220
225
230
235
240
245
250
255
Temperature °C
—■— solvent 50/50 0.1M NH$_4$OAc/CH$_3$OH m/z 335
—●— solvent 0.1 M NH$_4$OAc m/z 244

monitored, i.e. m/z 244 for 0.1 M ammonium acetate and m/z 335 for the 0.1 M ammonium acetate/methanol (50/50 v/v) solution. In 0.1 M ammonium acetate mobile phase the optimum tip temperature is 240° C, while in the 0.1 M ammonium acetate/methanol (50/50 v/v) solution the optimum tip temperature is 210° C. The decrease in the optimum tip temperature in the 0.1 M ammonium acetate/methanol (50/50 v/v) solution is conceivably responsible for the increase in the abundance of the pseudo-molecular ion of MC, as less heat is required for the thermospray vaporization/ionization of 0.1 M ammonium acetate/methanol (50/50 v/v).

Electrochemistry On -Line with Mass Spectrometry (EC/MS)

Figure 4.6 shows a schematic diagram of the on-line EC/TSP/MS system used to investigate the electrochemical reduction of MC, and used to monitor the guanosine adduct formation by the MC reductively activated in the electrochemical cell. The HPLC pump delivers the mobile phase and analyte first into the ESA electrochemical cell, where the on-line reduction of MC takes place. The analyte is then transferred to the thermospray interface, where the vaporization and the ionization of the mobile phase and the analyte is accomplished prior to mass analysis and detection by an electron multiplier.

Mass Spectrometric Hydrodynamic Voltammetry

Reactants, intermediates, and products of electrochemical reactions can be monitored as a function of electrode potential using EC/TSP/MS. This is done following the optimization of the thermospray conditions such as mobile phase composition and tip temperatures. Figure 4.7 shows the mass spectrometric hydrodynamic voltammogram (MSHV) of MC obtained by EC/TSP/MS with a mobile phase of 0.1M ammonium

Figure 4.6 Instrumental Setup for on-line EC/TSP/MS.

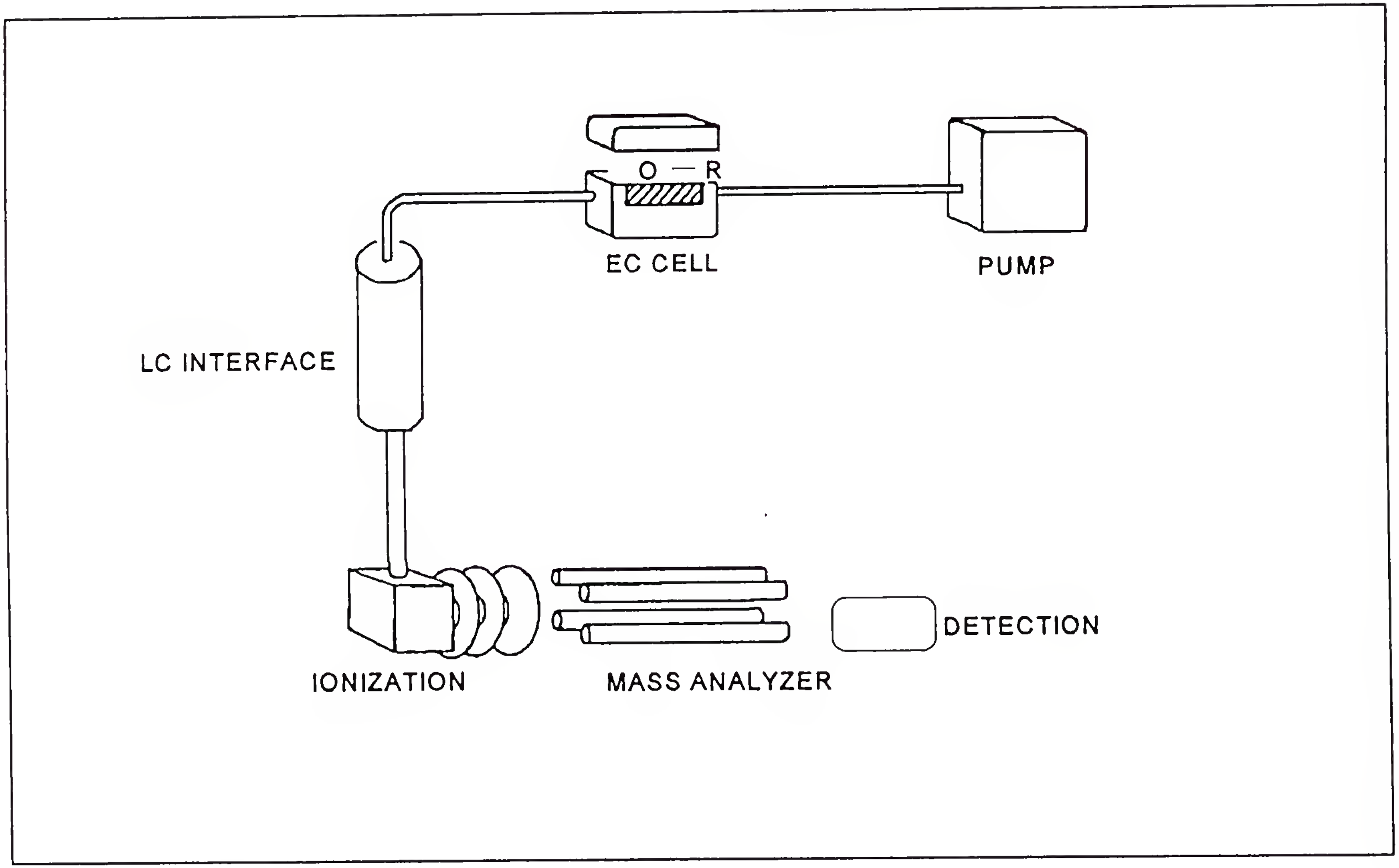
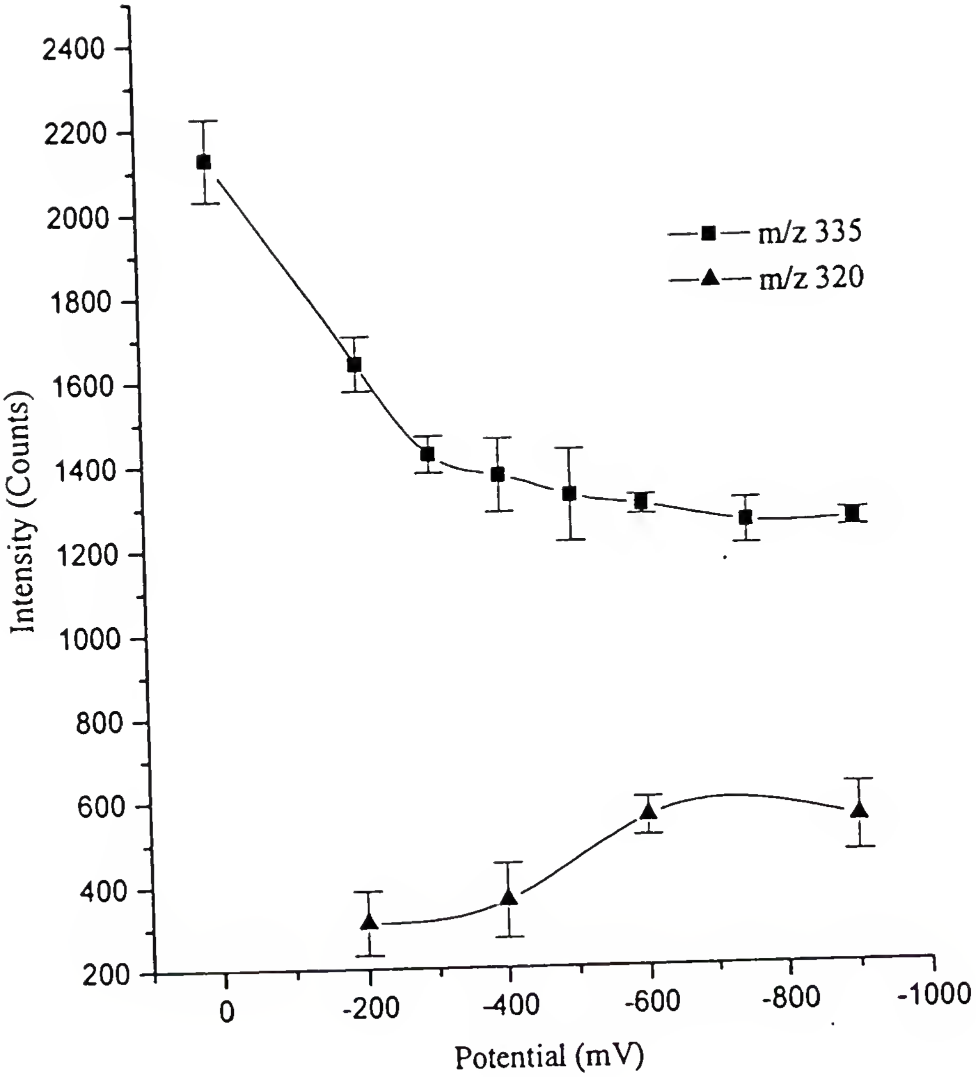

O — R
EC CELL
PUMP
LC INTERFACE
IONIZATION
MASS ANALYZER
DETECTION

Figure 4.7 Mass Spectrometric Hydrodynamic Voltammogram of Mitomycin C. Mobile Phase 50/50 0.1 M Ammonium Acetate/Methanol. Flow Rate 1.5 mL/min. Tip Temerature 210 °C.

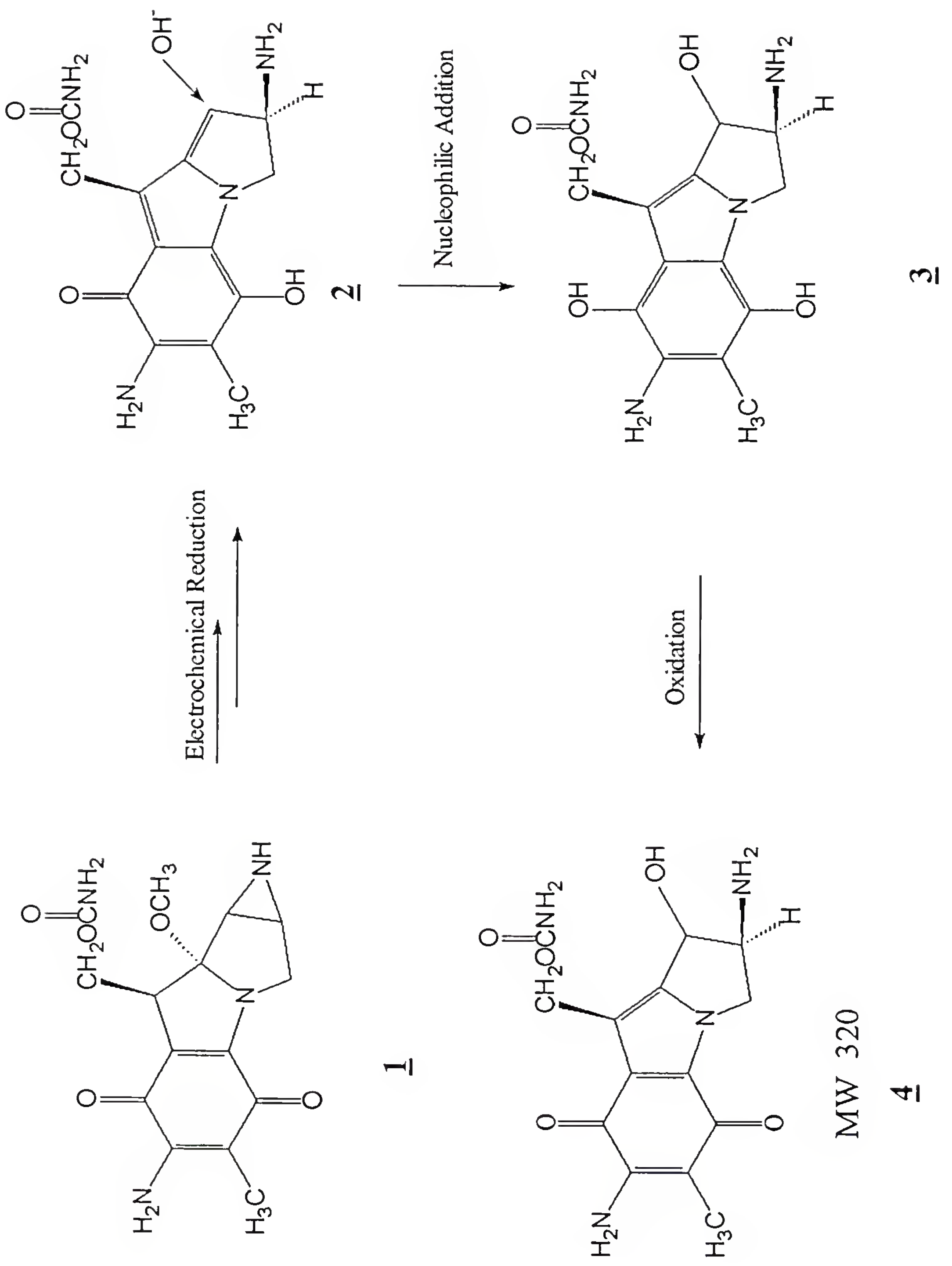

2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
Intensity (Counts)
0
-200
-400
-600
-800
-1000
Potential (mV)
—■— m/z 335
—▲— m/z 320

acetate/methanol (50/50 v/v) at a tip temperature of 210 °C at a potential range of 0.0 to 0.90 V. The on-line EC/TSP/MS reduction of MC is followed by monitoring the intensity of its pseudo-molecular ion $[M+H]^+$ at m/z 335 as a function of the potential applied to the working electrode in the electrochemical cell. The intensity of the m/z 335 ion decreases as the potential becomes more negative from 0 to -0.90 V. There is a corresponding increase in the intensity of an ion at m/z 320 which increases in correlation to the deceasing intensity of the m/z 335 ion. A plateau region begins at ca. -0.60 V for both curves. It has been shown that the plateau region which is observed here develops because of a steady-state behavior produced in all hydrodynamic voltammetry operating in the limiting current region.[80]

In the cyclic voltametric analysis of MC in phosphate buffer at pH 7.0 and in 0.1 M ammonium acetate at a glassy carbon (GC) electrode (Figure 5.7) the reduction potential of MC is -0.540 V vs SCE. Glassy carbon is a material with similar properties to the reticulated vitreous carbon working electrode material used in the on-line electrochemical cell. The reduction peak potential of MC obtained in off-line experiments, -0.560 V, should correspond to the potential of the steady-state region, of ca. -0.60 V. As shown by the results in Figure 4.7 the steady-state region is observed at the expected potential of ca. -0.60 V.

Figure 4.8 is a proposed scheme for the MC reaction as detected by the on-line EC/MS. The proposed pathway is also based on the results described in Chapter 3 for the off-line electrochemical experiments of MC and previously reported pathways for MC reduction in aqueous,[32] methanol [28] and DMA [35] solutions. As the potential is decreased MC is reduced in the electrochemical cell to its active form **2** ,which can undergo nucleophilic addition at the C-10 position with the hydroxy ion in solution acting as the nucleophile forming **3**. **3** can

Figure 4.8 Proposed Pathway of Mitomycin C Reactions in EC/TSP/MS.


1
Electrochemical Reduction
2
Nucleophilic Addition
3
Oxidation
MW 320
4
OH⁻
CH2OCNH2
OCH3
NH
NH2
OH
H2N
H3C

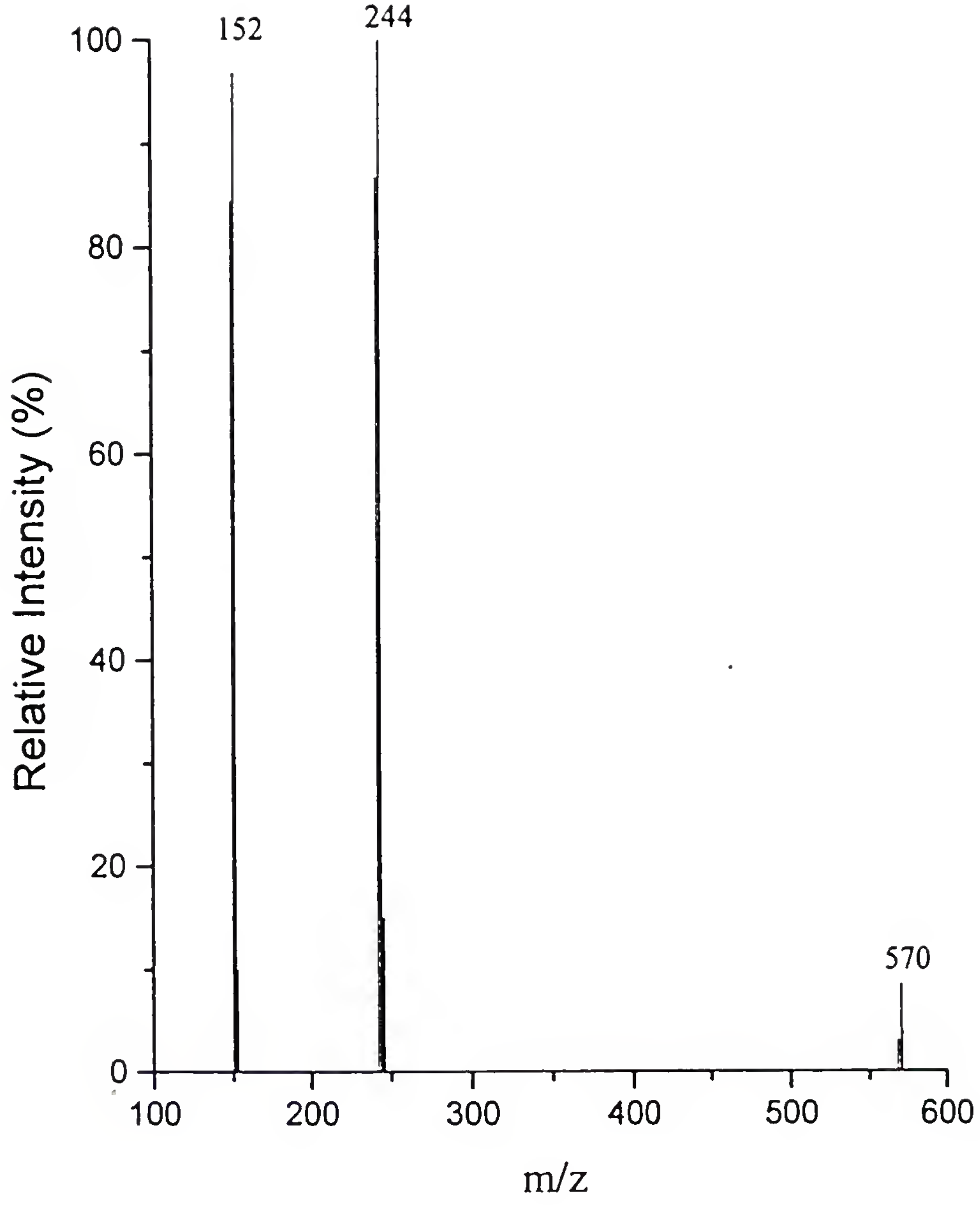
be oxidized by oxygen in solution forming **4** which is detected at m/z 320. In the MSHV (Figure 4.7), the decrease in the intensity of the MC pseudo-molecular ion at m/z 335 as the potentials become more negative corresponds to the reduction of MC forming possible the active form of MC **2** (Figure 4.8). The corresponding increase in the intensity of m/z 320 is due to the formation of the quinone **4** form of the hydroxy substituted **3**.

Formation of Mitomycin C Deoxyguanosine Adducts

The drug function of MC is exhibited *in vivo* after reductive activation by intracellular enzymes such as cytochrome-P450 reductase which is followed by alkylation of DNA by way of the DNA base, deoxyguanosine. In this work potential of using EC/TSP/MS to model the *in vivo* reactions of MC was investigated. A 100 to 1 molecular ratio of deoxyguanosine to MC solutions in 0.1 M ammonium acetate/methanol (50/50 v/v) was thoroughly mixed and then was introduced into the EC/TSP/MS system with a 0.1 M ammonium acetate/methanol (50/50 v/v) mobile phase. The concentration of deoxyguanosine was ten times greater that of MC to allow deoxyguanosine to compete effectively in adduct formation with other nucleophiles present in solution.

Figure 4.9 shows the mass spectrum obtained in the EC/TSP/MS of MC with deoxyguanosine in solution. The MC-deoxyguanosine adduct (MW 569) was detected as the pseudo-molecular $[M+H]^+$ ion at m/z 570 using selected ion monitoring following reductive activation of MC at -0.8 V. From the MSHV (Figure 4.7) on-line reduction of MC occurs at ca. -0.6 V. To allow for maximum conversion of MC to its active form a slight over potential of -0.8 V was used in these experiments. The peak at m/z 152 is due to the loss of the ribose group from deoxyguanosine to form the guanine base plus two protons. Figure 4.2

Figure 4.9 Mass Spectrum of EC/TSP/MS of Mitomycin C and Deoxyguanosine.
Electrochemical Cell at -0.8V.
Mobile Phase 0.1 M Ammonium Acetate\Metahol (50/50).
Tip Temerature 210 °C. Flow Rate 1.5 mL/min.


152
244
570
Relative Intensity (%)
0
20
40
60
80
100
100
200
300
400
500
600
m/z

shows the assignment of the fragments for the MC-deoxyguanosine adduct. Control experiments were also carried out with electrochemical cell off and the MC-deoxyguanosine adduct m/z 570 was not detected.

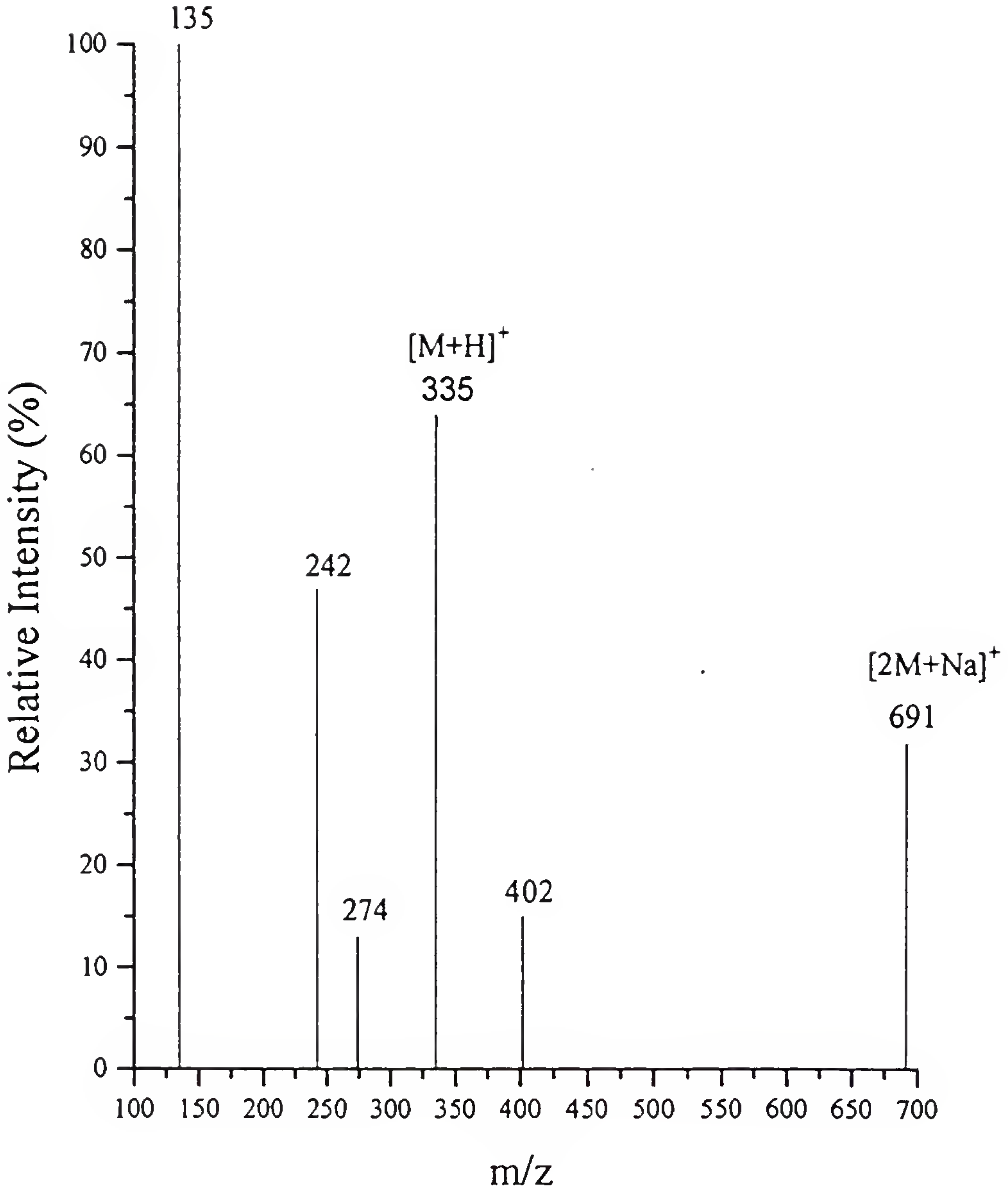
In the electrochemical cell MC can be reduced to its active form while deoxyguanosine which is electrochemically inert at the negative MC activation is unaffected. Deoxyguanosine then can act as a nucleophile and can react with the active form of MC.

On-Line Electrochemistry/Mass Spectrometry with an Electrospray Interface.

The fact that heat is applied during vaporization and ionization leads to limitations of thermospray as an interface for EC/MS. The elevated temperatures of the interface can degrade thermally liable intermediates and products of EC/MS experiments, complicating interpretation of the mass spectral data. This effect of heat was observed in the thermospray detection of MC pseudo-molecular ions, where at tip temperatures of 210 °C the pseudo-molecular ion m/z 335 was detected as the base peak (Figure 4.3), while at tip temperature of 240 °C this ion was in very low abundance or not detected. Studies were carried out to determine the viability of electrospray (ESP), which has a lower heat requirement was tested as an interface for EC/MS.

Although the results that are shown are preliminary, the full scan electrospray mass spectrum of MC (Figure 4.10) shows several interesting and characteristic features particularly since there is more fragmentation than was observed using the thermospray interface (Figure 4.3). In the electrospray MS the pseudo-molecular ion $[M+H]^+$ at m/z 335 is detected, but not as the base ion as in thermospray MS. The base ion in electrospray is at m/z 135. Assignment of electrospray fragment ions is shown in Table 4.1.

Figure 4.10 Electrospray Mass Spectrum of Mitomycin C.
Mobile Phase 50/50 Methanol/Water. Flow Rate 5.0 μl/min.


135
242
274
[M+H]$^+$
335
402
[2M+Na]$^+$
691
Relative Intensity (%)
0
10
20
30
40
50
60
70
80
90
100
100
150
200
250
300
350
400
450
500
550
600
650
700
m/z

The instrumental set-up used in the investigation of MC by EC/ESP/MS was similarto that shown in Figure 4.5 for EC/TSP/MS. The EC/ESP/MS mass spectrum of MC is shown in Figure 4.11. In these experiments because of the low flow rates (5 μL/min) and the relatively larger cell volume (50 μL) of the electrochemical cell bulk electrolysis was conducted for ca. two minutes at 0.80 V in the electrochemical cell prior to the delivery of the mobile phase into the electrospray interface. In the electrospray mass spectra which were obtained following the two minute bulk electrolysis, the pseudo-molecular ion $[M+H]^+$ at m/z 335 disappeared indicating that MC has been reduced. The m/z 135 ion which is still observed was shown in Figure 4.10 and has been previously discussed. The m/z 157 is the sodium adduct of the 135 ion. The ion at m/z 357 is the sodium adduct of MC. The appearance of an ion at m/z 301 in the EC/ESP/MS may indicate a possible reduction product of MC. Previous investigations have shown that when the solution conditions for the reduction of MC were moderately acidic (as they are for the electrospray investigations carried out here) the electrophilic adduct, 2,7-diaminomitosene,[81,82] MW 301, (Figure 4.12) predominates. This indicates that the ion at m/z 301 maybe the $M^{+\cdot}$ ion of 2,7-diaminomitosene.

Conclusions

From the results presented in this chapter the on-line EC/MS with a thermospray interface is shown as a feasible technique for modeling redox and the associated chemical reactions of MC relating to its drug function. EC/TSP/MS can be used to electrochemically activate MC, and detect the formation of the MC-deoxyguanosine adduct. The heat requirement of thermospray leads to several limitations of this interface such as the possible

Figure 4.11 Mass Spectrum of EC/ESP/MS of Mitomycin C following Reduction at -0.80 V in the Electrochemical Cell.
Mobile Phase 50/50 Methanol/Water. Flow Rate 5.0 μl/min.

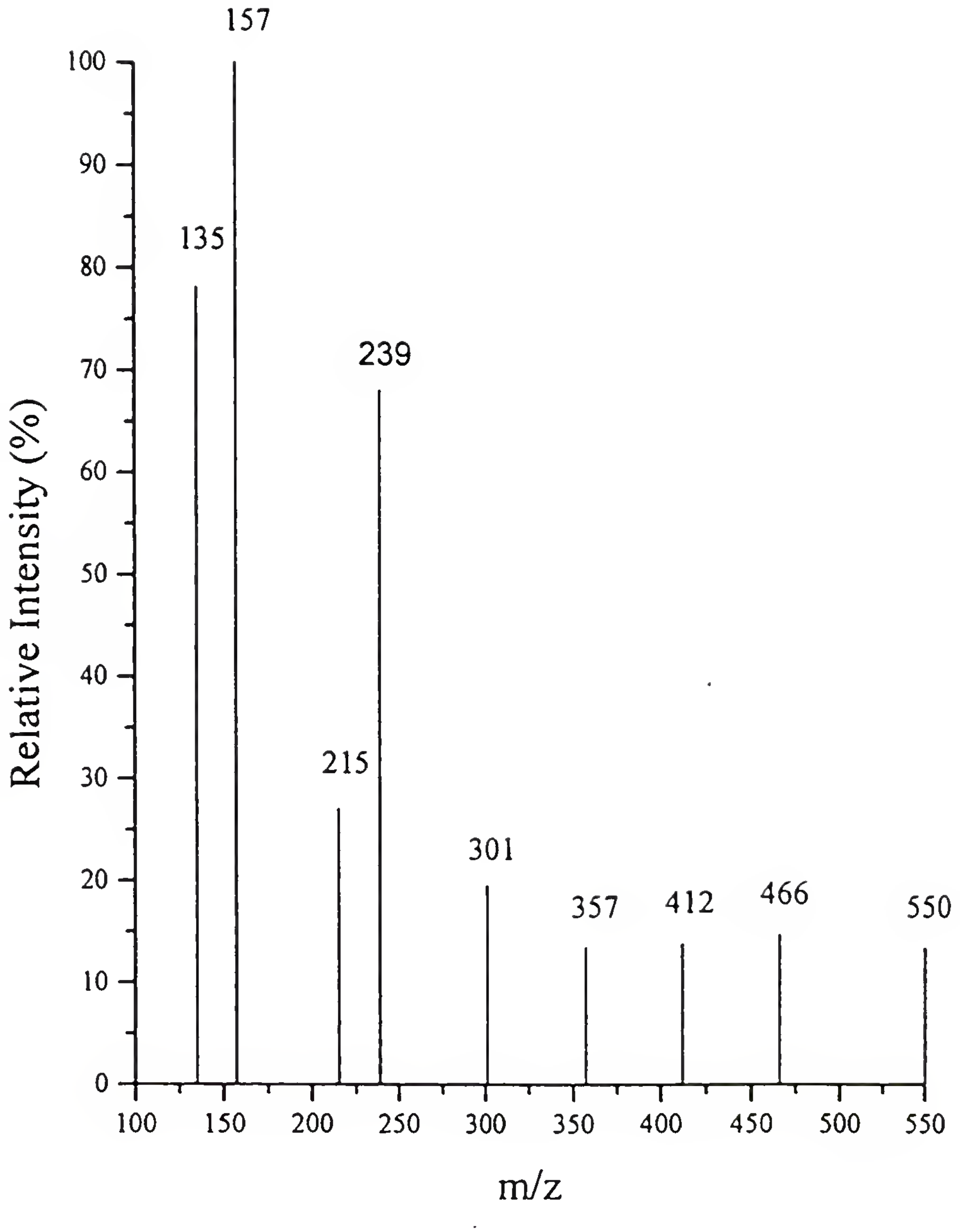
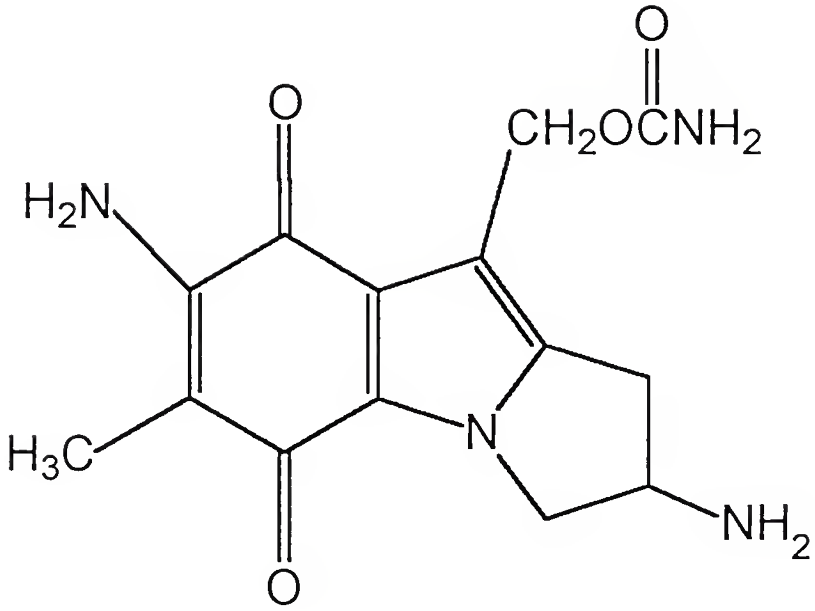

157
135
239
215
301
357
412
466
550
Relative Intensity (%)
0
10
20
30
40
50
60
70
80
90
100
100
150
200
250
300
350
400
450
500
550
m/z

Figure 4.12 Structure of 2,7-diaminomitosene MW 301. A Product of EC/ESP/MS of MC.



MW 301

degradation of the thermally liable intermediates and products of the electrochemical reactionsand the resulting low MS sensitivity. Electrospray which has a lower heat requirement was used for the on-line electrochemical reduction of MC to verify its capabilities.

The use of electrospray as an interface for EC/MS appears feasible in the detection of the reduction products of MC. The electrochemical cell used in present experiments has a volume of ca. 50 uL and the electrospray MS was operated at typical flow rates of 5.0 uL/min. This indicates that an electrochemical cell with a smaller volume will be needed for an optimum on-line EC/MS with an electrospray interface.

CHAPTER 5
DESIGN OF THIN LAYER CELL FOR ON-LINE ELECTROCHEMISTRY WITH MASS SPECTROMETRY

Introduction

One of the limitations of hydrodynamic voltammetry for on-line mass spectral measurements is the ability to develop an electrochemical cell with a working electrode that has long term stability and high electrochemical activity.[83] An additional requirement for the cell is the need for it to withstand high pressures up to ca. 1000 psi because of the back pressures from the thermospray interface in mass spectrometric detection. In addition, use of EC/MS in the investigations of biological redox reactions can be limited by electrode fouling resulting from irreversible adsorption of the analyte or reaction products at the electrode surface. This can lead to a gradual loss of sensitivity and eventual disappearance of response. The resurfacing or replacement of the working electrode in an electrochemical cell used for on-line EC/MS is then necessary. The commercial ESA electrochemical cell (Figure 4.1) used for on-line electrochemistry with mass spectrometry (EC/MS) is designed with a reticulated vitreous carbon (RVC) working electrode that allows no access to the working electrode for its resurfacing or replacement. As mentioned in Chapter 3 mitomycin C (MC) and its reduction products adsorb at graphite electrodes. With time, the RVC working electrode in the ESA electrochemical cell used in the EC/MS experiments with MC lost its electrochemical activity. This was attributed to the fouling of the working electrode by adsorption of MC reduction products some of which have known low solubility in water. In order to continue the work, the cell was replaced on two occasions.

In this chapter the development of an electrochemical cell for EC/MS based upon the thin layer cell (TLC) design is discussed. The cell was developed to simplify access to the working electrode for surface cleaning. The TLC design was used because of the small cell volume it provides. The cell was tested for its ability to withstand back pressures of ca. 1000 psi at typical flow rates of 1-2 mL/min.

The development of an electrochemical cell for EC/MS provides several challenges. The cell needs: 1) an easily removable working electrode which facilitates electrode resurfacing or replacement ; 2) to be able to tolerate the high back pressures of ca. 1000 psi at typical flow rates of 1.0-2.0 mL/min used with the thermospray interface; 3) a small dead volume to prevent the degradation of short-lived intermediates and loss of sensitivity and; 4) a large electrode area to volume ratio to allow a high conversion efficiency of the analyte. The demands 1,3 and 4 are met with the development of an electrochemical cell using a thin layer design. Achievement of demand 2 in the TLC design is also described.

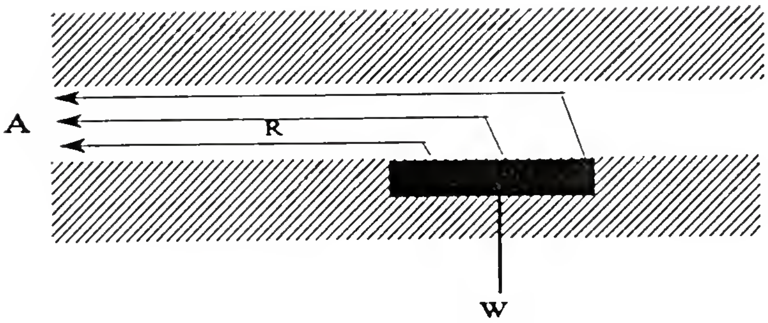
Thin Layer Cells

Thin layer cells have excellent flow characteristics and a high electrode surface to volume ratio. In the basic function of a thin layer cell the electroanalytical species undergoes a redox reaction as it passes over the electrode held at a constant potential sufficient for electron transfer.[84] The current response of the TLC is based on a specific case where the thickness of the cell is smaller than the thickness of the diffusion layer. In the TLC there is parallel flow of the analyte past the planar electrode and the boundary layer is constrained by the cell wall opposite the electrode.[85] Other types of thin layer cells using a porous carbon working electrode to improve conversion efficiency have been described.[86]

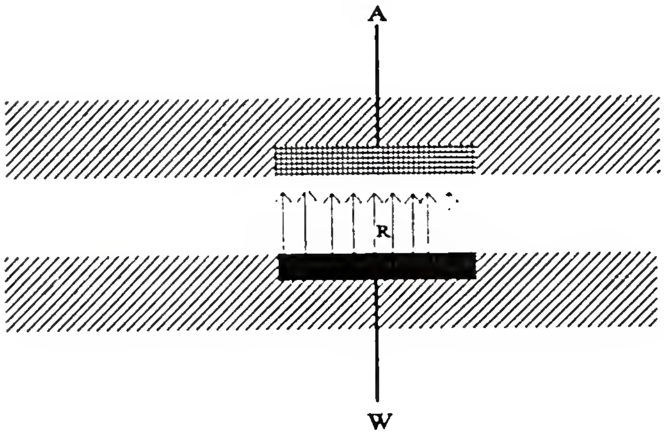
The iR drop is a common drawback associated with the operation of TLC. In high ionic strength aqueous solutions iR drop is less of a problem than in solvents with poor conductivity, such as non-aqueous solvents, where iR drop can be very severe. The iR drop is the ohmic potential drop in solution[1,2]. Its contribution to the applied electrode potential can be minimized by proper cell design. An additional problem in the operation of TLC is the non-uniform potential felt by the analyte at the working electrodes. In a TLC current between the working and auxiliary electrodes passes along the thin-layer channel (Figure 5.1a) if the auxiliary electrode is placed downstream to the working electrode. As a result the potential between the working electrode and solution is not uniform. The potentials upstream and downstream will differ due to the iR drop. To solve this problem the auxiliary electrode is placed perpendicular to the working electrode (Figure 5.1b). This design can reduce the iR drop to a negligible value and ensures that the potential is uniform at all points at the working electrode surface.

The typical thin layer cell designs used for many applications including high performance liquid chromatography (HPLC) detectors,[87] do not meet all the criteria (1 - 4) important for an electrochemical cell for EC/MS. In the typical design of the TLC design for electrochemical HPLC detection two blocks separated by a Teflon spacer are used to create the cell volume, and the thin layer cell severs as a salt bridge to the reference electrode which is typically placed down stream from the working electrode through a loose junction. The

Figure 5.1: IR Effects in Thin Layer Cells of Different Design:
a) Auxiliary Electrode is Downstream from the Working Electrode which Results in iR Drop.
b) Placement of the Auxiliary Electrode Across from the Working Electrode Minimizes iR Drop.



a



b

major limitation of this design is that high pressures are not tolerated and the reference electrode has a large dead volume which could lead to loss of sensitivity in the detection of the species of interest in the redox reactions.

Off-line Cyclic voltammetric investigations were done with a TLC with the design described above for an HPLC detector. The TLC design was modified by placing the auxiliary and reference electrodes directly perpendicular to working electrode. Figure 5.2 shows the cyclic voltammogram of potassium ferricyanide in 0.5 M phosphate buffer at scan rate of 10 mV/s. At this scan rate the response of potassium ferricyanide indicates iR is not a major contribution to cell response design.. At lower phosphate buffer concentrations of 0.05 M and higher scan rates response is not seen due to significantly higher iR drop effects.

Design of Electrochemical Cell for EC/MS

The choice of material to fabricate the electrochemical cell body was critical as an non-conductive material that could tolerate high pressures was required. The requirement of non-conductivity was to enable the electrical isolation of the three electrodes when the potential was applied to the cell. The polymer Nylon was chosen as the material for the cell body. The design of the electrochemical cell is shown in Figure 5.4. The auxiliary and reference electrodes are 0.1 mm diameter palladium wire which are inserted in a drilled hole perpendicular and directly opposite to the working electrode.

Figure 5.2: Cyclic Voltammogram of 10 mM Potassium Ferricyanide in 0.5 M Phosphate Buffer at Scan Rate of 10 mV/s in a Thin Layer Cell with a Glassy Carbon Working Electrode.

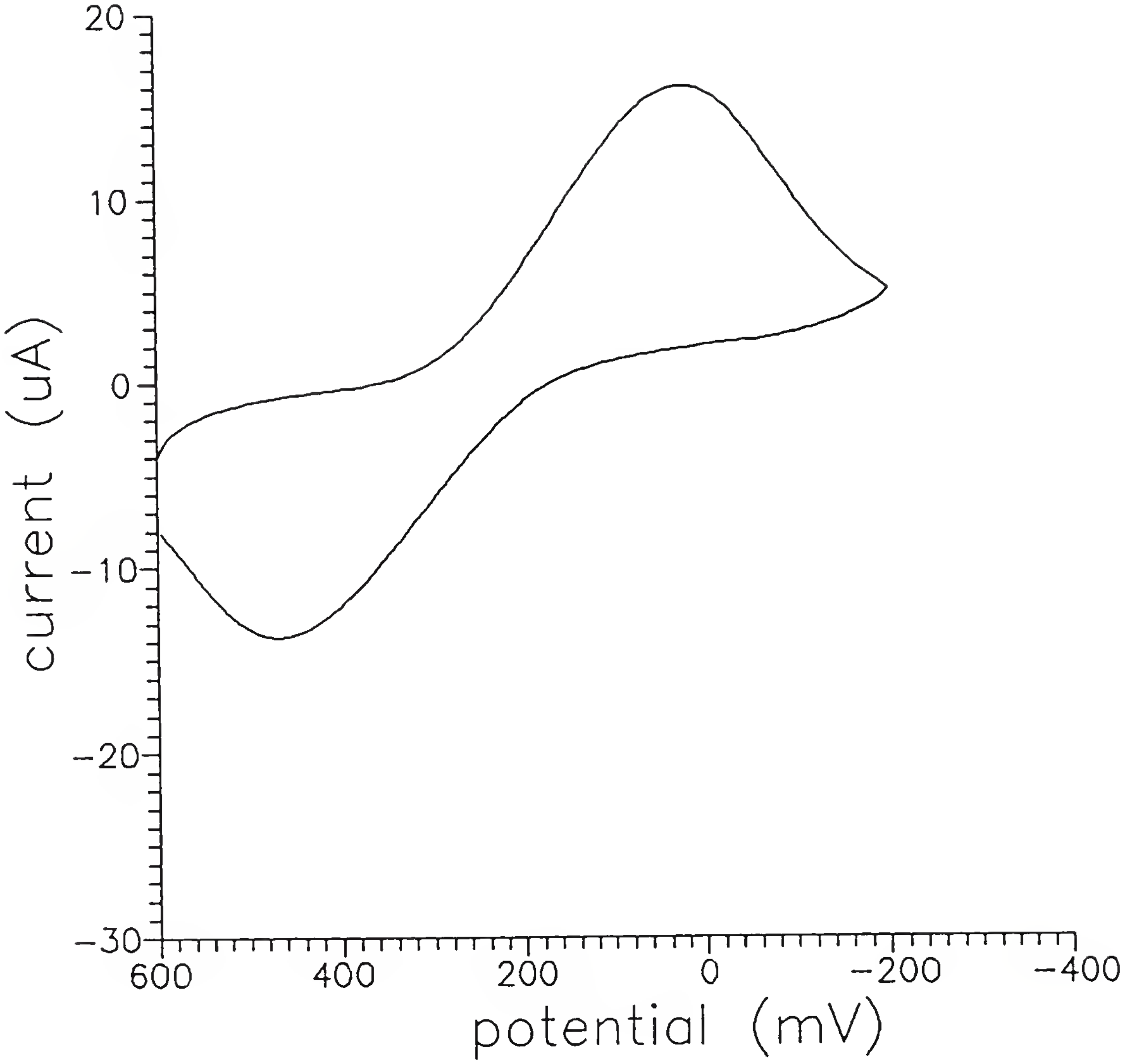
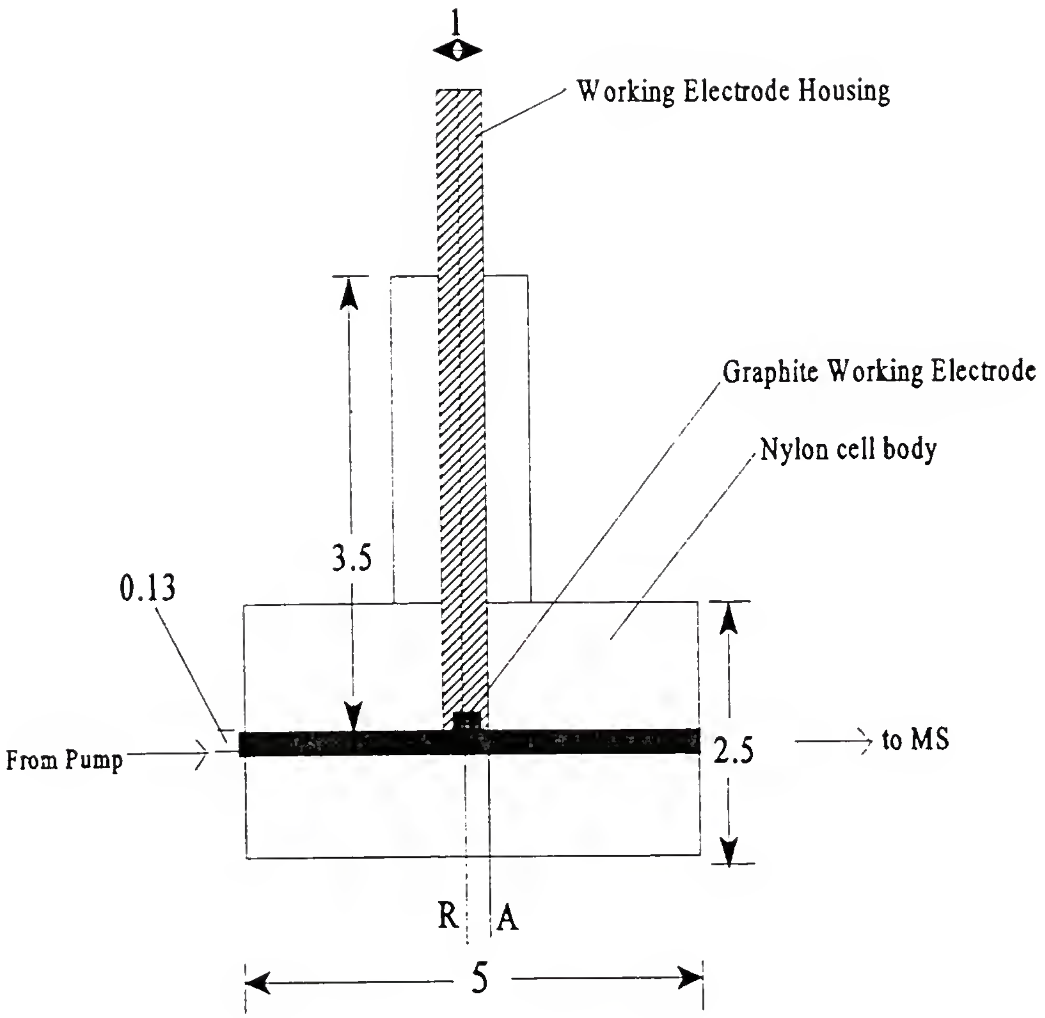

20
10
0
−10
−20
−30
current (uA)
600
400
200
0
−200
−400
potential (mV)

Figure 5.4 Design of Thin Layer Cell for On-Line Electrochemistry/Mass Spectrometry.
A = Auxiliary Electrode. (1 mm Pd wire)
R= Reference Electrode (1 mm Pd wire)
All Dimensionns in Figure in cm.


1
Working Electrode Housing
Graphite Working Electrode
Nylon cell body
3.5
0.13
From Pump
2.5
to MS
R
A
5

The working electrode housing can be screwed into the cell block. The threading iswrapped with Teflon tape to prevent leaking at high pressure. The geometric cell volume is ca. 80 µL when the working electrode is fully in place. To check that the working electrode is fully in place it can be electrically shorted with the perpendicular auxiliary electrode. The working housing electrode is then turned until there is no electric contact between the electrodes.

The "true" cell volume of an electrochemical flow cell is often significantly less than the geometric volume. This is because as the analyte band passes through the detector, the electrode reaction is confined to the thin layer adjacent to the electrode and not to the entire geometric cell volume.

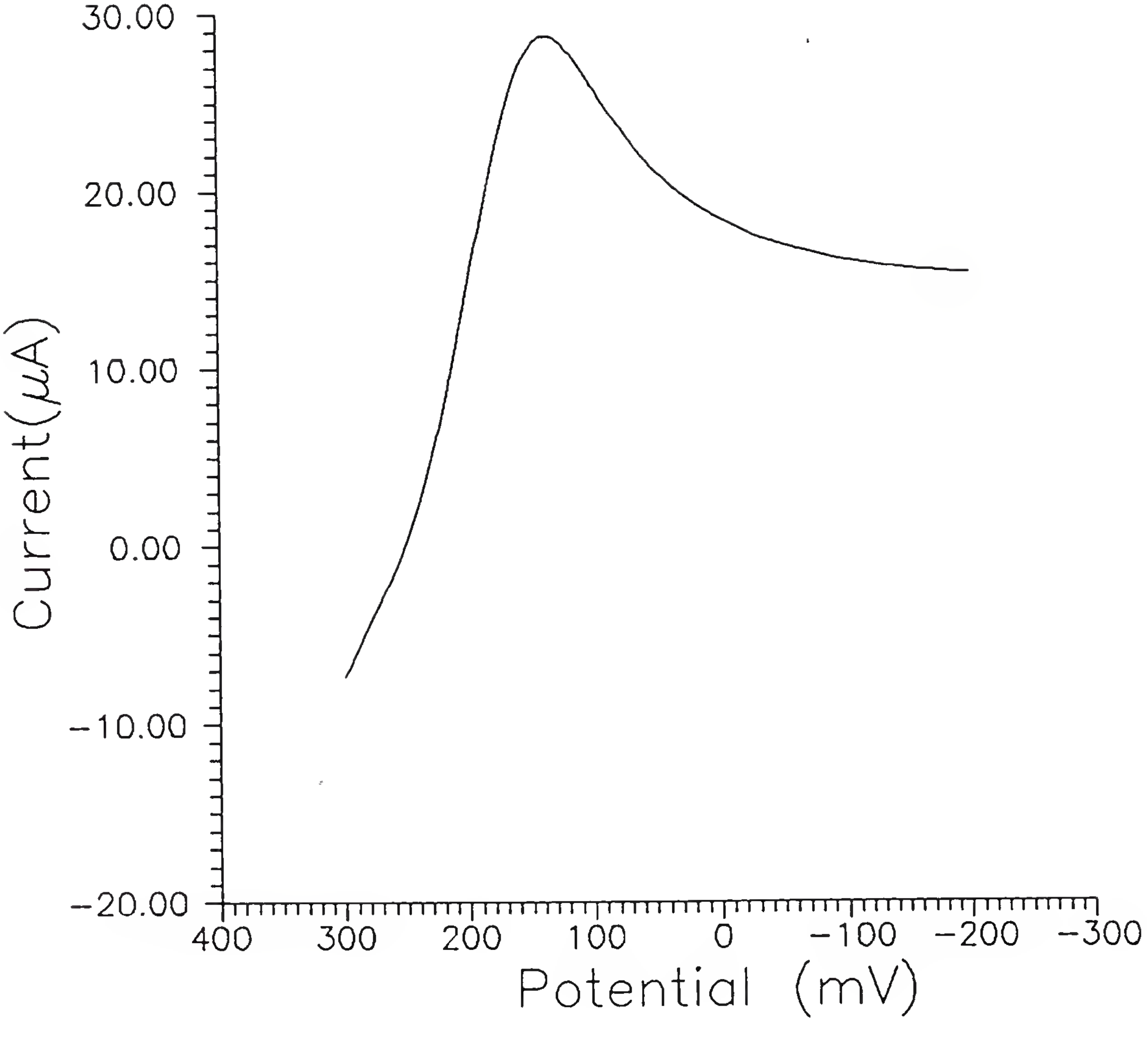
Fabrication of Working Electrodes

Graphite electrodes, glassy carbon (GC) and rough pyrolytic graphite (RPG) were fabricated for use with the thin layer electrochemical cell. Glassy carbon disk of diameter 3 mm or RPG of area ca. 0.05 cm^2 were sealed with inert epoxy in a hollow nylon housing. The electrical contact of the GC or RPG was made to copper wire with conducting silver epoxy. Prior to use, the working electrodes were resurfaced on 600-grit silicon carbide paper (Mark V Laboratory) using a polisher (Buehler Ecomet I) and rinsed with distilled deionized water. Glassy carbon was then polished with 0.1 µm gamma alumina particles (Gamal, Fisher Scientific Co.) and rinsed with distilled deionized water.

Off-Line Electrochemistry

Linear sweep voltammetry was used to characterize the thin layer cell. Figure 5.5 shows the voltammogram of potassium ferricyanide in bulk solution at the RPG electrode

Figure 5.5: Linear Sweep Voltammetry of 12 mM Potassium Ferricyanide on 0.1 M KCL at Scan Rate of 10 mV/s in Bulk Solution. Electrode Material RPG with an Area = 0.048 cm^2. The Electrode is Part of the Thin Layer Cell.


30.00
20.00
10.00
0.00
−10.00
−20.00
Current(μA)
400
300
200
100
0
−100
−200
−300
Potential (mV)

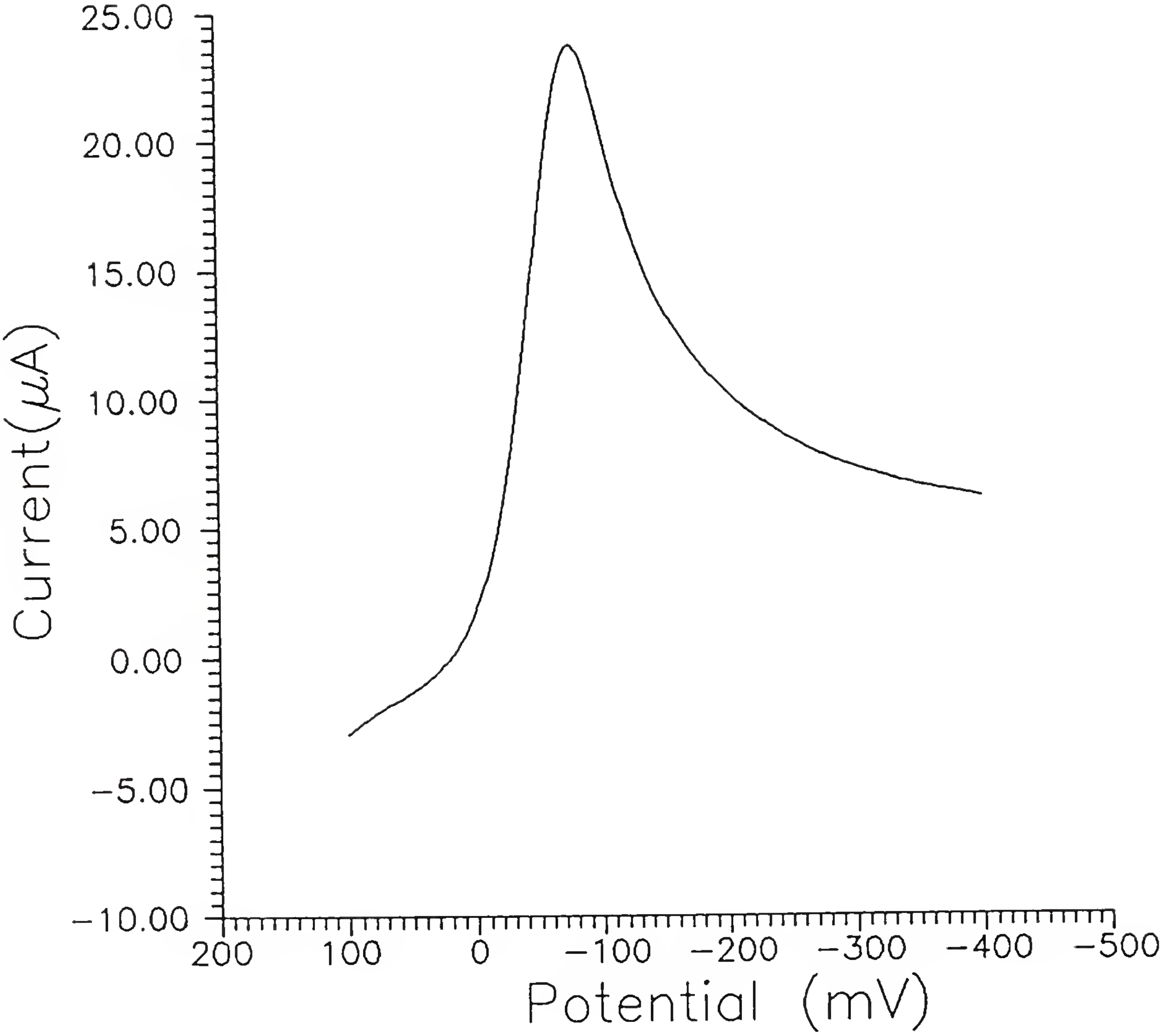
fabricated for the TLC. There is tailing of the peak which is expected for a diffusion controlled electron transfer system. Figure 5.6 shows the linear sweep voltammogram of potassium ferricyanide in the thin layer cell with the same RPG electrode as used in the bulk solution experiments. The sharp peak shape is characteristic of the thin layer behavior. The sharp peak is due to the fact that at this scan rate the cell thickness is less than the diffusion layer thickness. The contribution of diffusional mass transport from the solution bulk can be neglected, and consequently the analyte in the cell is depleted with results in the sharp drop-off compared to diffusionally tailing peaks..

On-Line Electrochemistry/Mass Spectrometry

On-line electrochemistry mass spectrometry was done to evaluate the effectiveness of the thin layer cell for the on-line mass spectrometric studies of electrochemical redox reactions. The electrochemical oxidation of uric acid has been previously characterized by EC/MS and uric acid was used as the model compound for these experiments. The detection of uric acid by thermospray mass spectrometry is difficult but one of its oxidation products, allantoin (MW 158), shows high sensitivity in thermospray mass spectrometry.

A mobile phase of 0.1 M ammonium acetate was used in the EC/MS for the evaluation of the thin layer electrochemical cell. A 100 µL sample of uric acid was injected into the mobile phase at a flow rate of 2.0 ml/min. When no potential was applied there was no allantoin detected by the mass spectrometer with selected ion monitoring for the $[M+H]^+$ m/z 159 after the uric acid was injected into the flowing stream. Allantoin was detected under similar conditions when the potential of the thin layer cell was held at 0.50 V where uric acid is expected to oxidize. This indicated that the cell functioned as expected in the

Figure 5.6: Linear Sweep Voltammetry of 12 mM Potassium Ferricyanide in 0.1 M KCL at Scan Rate of 10 mV/s in Thin Layer Cell. Electrode Material RPG with an Area = 0.048 $\mathrm{cm}^{2.}$


25.00
20.00
15.00
10.00
5.00
0.00
−5.00
−10.00
Current(μA)
200
100
0
−100
−200
−300
−400
−500
Potential (mV)

electrochemical oxidation of uric acid.

The back pressures of the thermospray interface was measured at ca. 1000 psi at flow rates of 2.0 ml/min. The back pressure is due to the exposure of the mobile phase to the vacuum system of the mass spectrometer. The thin layer electrochemical cell was able to withstand the back pressure experienced from the thermospray interface indicating that the cell design was compatible with the demands of EC/TSP/MS experiments.

Conclusions

The development of an electrochemical cell for on-line EC/TSP/MS with an accessible working electrode that can tolerate the high pressures of the thermospray interface (1000 psi) and has a small dead volume and large electrode area to volume ratio has been described. It has been shown that the placement of the auxiliary and reference electrodes are crucial in order to prevent iR drop across the electrode and to reduce of the dead volume of the cell.

The readily accessible working electrode allows for a periodic mechanical resurfacing of the electrode surface or replacement of the electrode material. Electrode materials suitable in analysis of specific redox systems can be used to optimize electrochemical response therefore, eliminating the problem of fouling of the working electrode that was experienced in the EC/MS investigations using the commercial (ESA) high pressure electrochemical cell.

CHAPTER 6

SUMMARY AND FUTURE WORK

In this dissertation the investigation of biological molecules by mass spectrometry and electrochemistry has been described. Amino acids were analyzed by GC/MS and the anti-cancer drug mitomycin C was investigated by electrochemistry on-line with mass spectrometry as well as off-line electrochemistry to gain insights into its reactivity.

In Chapter 2 the feasibility of simultaneous acylation and esterification of 21 amino acids using perfluorinated acid anhydrides and fluorinated alcohols was investigated. For 15 of the amino acids investigated the step single step unambiguous derivatives were formed. In each case ECNI produces a base-peak ion characteristic of the amino acid which makes it , therefore, suitable for detection by selected ion monitoring (SIM).

The nine amino acids with unreactive side chains along with serine, threonine aspartic acid, lysine, tyrosine and arginine produce analytically useful derivatives. Of the remaining six amino acids asparagine, glutamine and cystine produce contradictory derivatives, as the amino acids are converted to other amino acids during the derivatization reaction. Glutamic acid, tryptophan and cysteine form multiple ECNI sensitive products, which limits the use of a single step acylation/esterification for analysis of these amino acids.

The single step acylation/esterification is impractical for total amino acid analysis of protein hydrolysates, but can provide rapid analysis and high sensitivity for the detection of specific amino acids.

Chapter 3 describes the electrochemical investigations of mitomycin C. The electrochemical analysis of MC reduction in aqueous and methanol solutions at graphite electrodes is described. It was confirmed from the electrochemical studies in methanol solutions at graphite electrodes that the reduction of MC consists of an electrochemical-chemical-electrochemical reaction. The chemical reaction represents one or more steps. The electrochemical steps are considered reversible while the chemical steps are considered irreversible. The electrochemical activation of MC is followed by a series of irreversible chemical reactions including the addition of a nucleophile.

The electrochemical studies of MC in methanol suggest that the initial one-electron reduction step, leads to the subsequent loss of methanol and the opening of the three-membered arizidine ring and therefore, the chemical changes proceed from the quinone radical stage. Cyclic voltammetric analysis of MC in aqueous solutions at graphite electrodes suggests that the initial reduction of MC is to the hydroquinone.

Adsorption of the reduction product of MC was observed at RPG electrodes in aqueous solutions. This was confirmed by several experiments. Surfactants were used to probe the electrode surface behavior as it has been shown in previous work by this group that the addition of an ionic surfactant to a solution containing an adsorbing electroactive species can decrease the adsorption of that species at graphite electrodes. Scan rate studies also confirmed adsorption at RPG electrodes.

It was important to verify the nature of the MC interactions with the electrode surface because the electrochemical cell used in on-line EC/MS investigations uses graphite as the working electrode. Adsorption of MC reduction products at the electrode surface can eventually foul the electrode and lead to loss of reactivity, which could lead to false interpretation of the MS results.

In Chapter 4 the electrochemistry on-line with mass spectrometry (EC/MS) of MC is described. From the results presented in this chapter the on-line EC/MS with a thermospray interface is shown as a feasible technique for modeling redox and the associated chemical reactions of MC relating to its drug function. EC/TSP/MS can be used to electrochemically activate MC, and detect the formation of the MC-deoxyguanosine adduct.

The use of electrospray as an interface for EC/MS appears feasible in the detection of the reduction products of MC. The major advantage of electrospray over thermospray as an interface for EC/MS is its lower heat requirement. The higher heat required for thermospray could lead to possible degradation of the thermally liable intermediates and products of the electrochemical reactions resulting in low MS sensitivity.

In Chapter 5 the development of an electrochemical cell for on-line EC/TSP/MS, with an accessible working electrode, that can tolerate the high pressures of thermospray interface (1000 psi) was described. This electrochemical cell design was based on a thin layer cell. In the cell design the auxiliary and reference electrodes were placed perpendicular to the working electrode to reduce iR drop. The electrochemical cell has a working electrode which can be accessed for resurfacing or replacement. This feature is a major advantage over electrochemcal cells previously used for EC/MS, where the working electrode was sealed and over time became inactive due to fouling of the surface.

The continued development of the thin layer cell is critical. The conversion efficiency of the cell could be increased by increasing the area of the working electrode to the cell volume. This could be accomplished by replacing the working electrode with a porous electrode.

Future work in EC/MS will include the continued development of electrospray as an interface. The advantages of the electrospray interface over thermospray is the lower heat requirement described in Chapter 4 along with the extended mass range of electrospray which allows for the detection of large biological molecules. Typical electrospray flow rates in the µL/min range require an electrochemical cell with a sub-µL volume.

Other future directions of on-line EC/MS is in the detection of carcinogens. Many carcinogens initiate there effects by reacting at electron-rich sites on DNA only after *in vivo* redox activation by biological compounds. Electrochemistry could be used to model the *in vivo* redox activation and on-line mass spectrometry can be used to the detect the adduct formation.

BIBLIOGRAPHY

1. Voet, D.; Voet, J. G. *Biochemistry*; John Wiley and Sons: New York, 1989; Chapter 4.

2. Lehniger, A. L. *Biochemistry*; Worth Publishers: New York, 1985.

3. Bremer, H. J. *Disturbances of Amino Acid Meatbolism: Clinical Chemistry and Diagnosis*; Urban and Schwarzenberg: Baltimore, 1981.

4. Fox, A.; Ueda, K.; Morgan, S. In *Analytical Microbiological Methods: Chromatography and Mass Spectrometry*; Fox, A.; Morgan, S.; Larsson, L.; Oldham, G.; Ed; Plenum Press: New York, 1984; Chapter 8.

5. Vetter, W. In *Biomedical Applications of Mass Spectrometry*; Waller, G. R.; Dermer, O. C.; Ed.; Wiley, Interscience: New York, 1980; Chapter 14.

6. Lawson, A. M. In *Mass Spectrometry*; Lawson A. M.; Ed.; Walter deGruyter: New York, 1989.

7. Winkler, H. U.; Beckeyl, D. *Org. Mass Spectrom.* **1972,** *6*, 655.

8. Husek, P.; Macek, K. *J. Chromatogra.* **1975,** *113*, 139.

9. Leimer, K. R.; Rice, R. H.; Gehrke, C.W. *J. Chromatogra.* **1977,** *141*, 121.

10. Gehrke, C. W.; Nakamoto, H.; Zumwalt, R. W. *J. Chromatogra.* **1969,** *45*, 24.

11. Kasier, F. E.; Gehrke, C. W. Zumwalt, R. W.; Kuo, K.C. *J. Chromatogra.* **1974,** *94*, 113.

12. Kingston, E. E.; Duffield, A. M. *Biomed. Mass Spectrom.* **1978,** *5*, 621.

13. Bourne, E. J.; Stacey, S.; Tatlow, J. C.; Tedder, J. M. *J. Am. Chem. Soc.* **1949,** *72*, 2967.

14. Bourne, E. J.; Randles, J. E. B.; Stacey, S.; Tatlow, J. C.; Tedder, J. M. *J. Am. Chem. Soc.* **1954,** *76*, 2967.

15. Toth, J. P.; Gregory III, J. F. *Biomed. Environ. Mass Spectrom.* **1988,** *17*, 73.

16. Wilk, S.; Orlowski, M. *Anal. Biochem.* **1975,** *69*, 100.

17. Hunt, D. F.; Crow, F. W. *Anal. Chem.* **1978,** *50*, 1781.

18. Christophorou, L. G. *Environmental Health Perspectives* **1980,** *36*, 3.

19. Dougherty, R. *Anal. Chem.* **1981,** *53*, 625A.

20. Hass, J. R.; Friesen, M. D.; Harvan, D. J.; Parker, C.E. *Anal. Chem.* **1978,** *50*, 1474.

21. Low, G. K.-C.; Duffield, A. M. *Biomed. Mass Spectrom.* **1984,** *11*, 223.

22. Hata, T.; Sano, Y.; Sugawara, R.; Matsumae, A.; Kanamori, K.; Shima, T.; Hoshi, T. *J. Antibiot. Ser. A* **1956,** *9*, 141.

23. Wakaki, S.; Marumo, H.; Tomoika, K.; Shimizu, G.; Kato, E.; Kamada, H.; Kudo, S.; Fujimoto, Y. *Antibiot. Chemother.* **1958,** *8*, 228.

24. Beijen, H. B.; Bult, A.; Underberg, J. M. *Analytical Profiles of Drug Substances* **1987,** *16*, 361.

25. Tomasz, M.; Lipman, R. *Biochemistry* **1981,** *20*, 5056.

26. Andrews, P. A.; Pan, S-S.; Bachur, N. R. *J. Am. Chem. Soc.* **1986,** *108*, 4158.

27. Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G.; Nakanishi, K. *Science* **1987,** *235*, 1204.

28. Kohn, H.; Zein, N.; Lin, X. Q.; Ding, J.-Q.; Kadish, K. M. *J. Am. Chem. Soc.* **1987,** *109*, 1833.

29. Iyer, V. N.; Szybalski, W. *Science* **1964,** *145*, 55.

30. Moore, H. W. *Science* **1977,** *197*, 527.

31. Musser, S. M.; Pan S. -S.; Callery, P. S. *J. Chromatogra.* **1988,** *474*, 197.

32. Rao, G. M.; Begleiter, A.; Lown, J. A. *J. Electrochem. Soc.* **1977,** *124*, 199.

33. Ryun, C. H.; Park, S. M. *J. Electrochem. Soc.* **1985,** *132*, 2426.

34. Dryhurst, G.; Kadish, K. M.; Schellur, F.; Renneberg, R. *Biological Electrochemistry;* Academic Press: New York, 1982.

35. Andrews, P. A.; Pan, S-S.; Bachur, N. R. *J. Am. Chem. Soc.* **1986,** *108*, 4158.

36. Mckenna, K.; Brajter-Toth, A. *J. Electroanal. Chem.* **1987,** *233*, 49.

37. Miner, D. J.; Rice, J. R.; Riggin, R. M.; Kissenger, P. T. *Anal. Chem.* **1981,** *122,* 205.

38. Brajter-Toth, A.; Dryhurst, G. *J. Electroanal. Chem.* **1981,** *122*, 205.

39. Blakely, C. R.; Vestal, M. L. *Anal. Chem.* **1983,** *55*, 750.

40. Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985,** *57*, 675.

41. Vestal, M. L.; Fergusson, G. L. *Anal. Chem.* **1985,** *57*, 2373.

42. Volk, K. J.; Yost. R. A.; Brajter-Toth, A. *Anal. Chem.* **1992,** *64*, 21A.

43. Hambitzer, G.; Heitbaum, J. *Anal. Chem.* **1986,** *58, 1067.*

44. Volk, K. J.; Yost. R. A.; Brajter-Toth, A. *Anal. Chem.* **1989,** *61*, 1709.

45. Meng, C. K.; Mann, M.; Fenn, J. B. *J. Phys. D.* **1988,** *10*, 368.

46. Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Baringa, C. J.; Udseth, H. R. *Anal. Chem.* **1990,** *62*, 882.

47. Toth, J. P.; Moye, H. A.; In *Applications of New Mass Spectrometry Techniques in Pesticide Chemistry,* Rosen, J.; Ed.; John Wiley and Sons: New York, 1987.

48. Deyrup, C. L.; Chang, S. M.; Weintraup, R. A.; Moye, H. A. *J. Agr. Food. Chem.* **1985,** *33*, 942.

49. Roberts, M. B. V. *Biology: A Functional Approach*; Thomas Nelson and Sons: Surrey, Great Britian, 1980.

50. Corso, G.; Esposito, M.; Gallo, M.; Dellorusso, A.; Antonioni, M. *Bio. Mass. Spectrom.* **1993,** *22*, 698.

51. Weissbach, A.; Lisio, A. *Biochemistry* **1965,** *4*, 1966.

52. Dorr, R. T.; Bowden, G. T.; Alberts, D. S.; Liddil, J. D. *Cancer Res.* **1985,** *45*, 3510.

53. Iyer, V. N.; Szybalski, W. *Proc. Natl. Acad. Sci. U.S.A.* **1963,** *50*, 353.

54. Meyn, R. E.; Jenkins, S.F.; Thompson, L.H. *Cancer Res.* **1982,** *42*, 3106.

55. Schwartz, H.S.; Sodergen, J. E.; Phillips, F. S. *Science* **1963,** *142*, 1181.

56. Pan, S.; Andrews, P. A.; Glover, C. J.; Bachur, N. R. *J. Biol. Chem. Soc.* **1984,** *259*, 959.

57. Keyes, S. R.; Fracasso, P. M.; Heimbrook, D. C. Rockwell, S.; Sliger, S. G.; Sartorelli, A. C. *Cancer Res.* **1984,** *44*, 5638.

58. Hoey, B. M.; Butler, J.; Swallow, A. J. *Biochemistry* **1988,** *27*, 2608.

59. Lown, J. W. In *Mitomycin C- Current Status and New Developments*; Carter, S. K.; Crooke, S. T.; Eds; Academic: New York, 1979, pp 5-26.

60. Tomasz, M.; Mercado, C. M.; Olson, J.; Chatterjie, N. *Biochemistry* **1974,** *13*, 4878.

61. Rao, G. M.; Begleiter, A.; Lown, J. A. *J. Electrochem. Soc.* **1977,** *124*, 195.

62. Kinoshita, K. *Carbon: Electrochemical and Physicochemical Properties*; John Wiley and Sons: New York, 1988.

63. McCreey, R. L. In *Electroanalytical Chemistry*; Bard, A. J.; Ed; Dekker: New York, 1991, Vol 17, 221-373.

64. Marino, A. Ph.D. Thesis, University of Florida, 1994.

65. Bodalbhai, L.; Brajter-Toth, A. *Anal. Chim. Acta.* **1990,** *231*, 191.

66. Stackelberg, M.; Pilgram, M.; Toome V. *Z. Electrochem.* **1953,** *57*, 342.

67. Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications;* John Wiley and Sons: New York, 1980.

68. Ramette R. W. *Chemical Equilibrium and Analysis*; Addison-Wesley: London, 1981, Chapter 11.

69. Dryhurst, G.; Kadish, K. M.; Schellur, F.; Renneberg, R. *Biological Electrochemistry;* Academic Press: New York, 1982. Chapter 1.

70. Marino, A.; Brajter-Toth, A. *Anal. Chem.* **1992,** *65*, 370.

71. Wopscall, R. H.; Shain, I. *Anal. Chem.* **1967,** *39*, 1512.

72. Nicholson, R. S.; Shain, I. *Anal. Chem.* **1964,** *36*, 706.

73. Eggins, B. R.; Chambers, J. Q.; *J. Electrochem. Soc.* **1970,** *17*, 186. Jaworski, J. S.; Lesniewska, E.; Kalinowski, M. K. *J. Electrochem. Soc.* **1979,** *105*, 329. Kamel, L. A.; Shams El Din, A. M. *J. Electrochem. Soc.* **1970,** *24*, 230. and references therein.

74. Danishefsky, S. J.; Egbertson, M. *J. Am. Chem. Soc.* **1986,** *108*, 4648.

75. Kuwana, T.; Winograd, N. *Electroanalytical Chemistry*; Marcel Dekker: New York, 1974: Vol 7, Chapter I.

76. Dryhurst, G.; Kadish, K. M.; Schellur, F.; Renneberg, R. *Biological Electrochemistry;* Academic Press: New York, 1982.

77. Volk, K. Ph.D. Disseration University of Florida 1989.

78. Getek, T. A.; Korfmacher, W. A.; Mcrae, T. A.; Hinson J. A. *J. Chromatogra.* **1989,** *474,* 245.

79. Van Lear, G. E. *Tetrahedron*, **1970,** *26*, 2587.

80. Kissinger, P. T.; Heinemann, W. R. *Laboratory Techniques in Electroanalytical Chemistry;* Marcell Dekker, Inc.: New York, 1984.

81. Schiltz, P.; Kohn H. *J. Am. Chem. Soc.* **1992,** *114,* 7958.

82. Han, I.; Russell, D. J.; Kohn, H.*J. Org. Chem.* **1992,** *52,* 1799.

83. Stulik, K.; Pacakova, V. *Electroanalytical Measurements in Flowing Liquids;* Ellis Horwood: Chichester, 1987.

84. Kissenger, P. T. In *Laboratory Techniques in Electroanalytical Chemistry;* Kissinger, P. T.; Heinemann, W. R.; Eds; Marcell Dekker, Inc.: New York, 1984.

85. Hubbard, A.T.; Anson, F. C.In *Electroanalytical Chemistry* Bard, A. J.; Ed.; Marcel Dekker: New York, 1971.

86. Davis, B. K.; Weber, S. G. *Anal. Chem.* **1994,** *66,* 1204,

87. Gunasingham, H.; Fleet, B. In *Electroanalytical Chemistry* Bard; A. J.; Ed.; Marcel Dekker: New York, 1989 Vol 16.

BIOGRAPHICAL SKETCH

Maurice Vincent Wayne Thompson was born in St. Andrew, Jamaica, on January 7, 1966. He is the youngest of five children of Vincent and Sylvia Thompson. After graduating from Calabar High School in 1982, Maurice attended the University of the West Indies, Mona, Jamaica. In January 1985 he transferred to the University of Florida where he earned a Bachelor of Science degree in chemistry in August 1987.

In January 1990, Maurice began his graduate studies in analytical chemistry at the University of Florida. While completing of his graduate studies he accepted a position as Research Associate at International Paper in Tuxedo, New York.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Anna Brajter-Toth, Chair
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James D. Winefordner
Graduate Research Professor of
Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Powell
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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